Paul R. Ortiz de Montellano *Editor*

Cytochrome P450

Structure, Mechanism, and Biochemistry

Fourth Edition

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Editor
Paul R. Ortiz de Montellano
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About the Editor

Paul R. Ortiz de Montellano received his PhD in bioorganic chemistry from Harvard University, Cambridge, MA. After postdoctoral work as a North Atlantic Treaty Organization Fellow in Zürich, Switzerland, and a stint with Syntex in Mexico City and Palo Alto, California, he joined the faculty of the University of California in San Francisco, where he is currently Professor, Vice-chair of the Department of Pharmaceutical Chemistry, and Associate Dean for Research of the School of Pharmacy. His research interests center on the structure, mechanism, inhibition, and biochemistry of hemoproteins, including the cytochrome P450 enzymes. He has received the B.B. Brodie Award in Drug Metabolism from the American Society of Pharmacology and Experimental Therapeutics, the R.T. Williams Distinguished Scientific Achievement Award from the International Society for the Study of Xenobiotics, and the Ernest H. Volwiler Research Achievement Award from the American Association of Colleges of Pharmacy.

Contributors

Richard J. Auchus Division of Metabolism, Endocrinology, and Diabetes, Department of Internal Medicine, University of Michigan Medical Center, Ann Arbor, MI, USA

Patrick J. Bakkes Institute of Biochemistry, Heinrich Heine University Düsseldorf, Düsseldorf, Germany

Thomas K. H. Chang Faculty of Pharmaceutical Sciences, The University of British Columbia, Vancouver, BC, Canada

Jennifer Cheng Division of Intramural Research, National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA

Maria Almira Correia Department of Cellular and Molecular Pharmacology, University of California, San Francisco, CA, USA

Ilia G. Denisov Department of Biochemistry, The School of Molecular and Cellular Biology, University of Illinois, Urbana, IL, USA

Matthew L. Edin Division of Intramural Research, National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA

Marco Girhard Institute of Biochemistry, Heinrich Heine University Düsseldorf, Düsseldorf, Germany

Saki Gotoh Pharmacogenetics Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC, USA

Artiom Gruzdev Division of Intramural Research, National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA

F. Peter Guengerich Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, TN, USA

Paul. F. Hollenberg Department of Pharmacology, University of Michigan, Ann Arbor, MI, USA

Samantha L. Hoopes Division of Intramural Research, National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA

Eric F. Johnson Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA, USA

Kaname Kawajiri Research Institute for Clinical Oncology, Saitama Cancer Center, Ina-machi, Saitama, Japan

J.-J. Kim Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI, USA

David Leys Faculty of Life Sciences, Manchester Institute of Biotechnology, The University of Manchester, Manchester, UK

Osama Mahmoud Institute of Biochemistry, Heinrich Heine University Düsseldorf, Düsseldorf, Germany

Kirsty J. McLean Faculty of Life Sciences, Manchester Institute of Biotechnology, The University of Manchester, Manchester, UK

Walter L. Miller Division of Endocrinology, Department of Pediatrics, University of California, San Francisco, CA, USA

Andrew W. Munro Faculty of Life Sciences, Manchester Institute of Biotechnology, The University of Manchester, Manchester, UK

Masahiko Negishi Pharmacogenetics Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC, USA

Marumi Ohno Pharmacogenetics Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC, USA

Paul R. Ortiz de Montellano Department of Pharmaceutical Chemistry, University of California, San Francisco, CA, USA

Thomas L. Poulos Departments of Molecular Biology and Biochemistry, Pharmaceutical Sciences, and Chemistry, University of California, Irvine, CA, USA

Mary A. Schuler Department of Cell and Developmental Biology, University of Illinois, Urbana, IL, USA

Stephen G. Sligar Department of Biochemistry, The School of Molecular and Cellular Biology, University of Illinois, Urbana, IL, USA

Vlada B. Urlacher Institute of Biochemistry, Heinrich Heine University Düsseldorf, Düsseldorf, Germany

Lucy Waskell University of Michigan Medical School and VA Medical Center, Ann Arbor, MI, USA

David J. Waxman Division of Cell and Molecular Biology, Department of Biology, Boston University, Boston, MA, USA

Kouichi Yoshinari Department of Molecular Toxicology, School of Pharmaceutical Sciences, University of Shizuoka, Suruga-ku, Shizuoka, Japan

Darryl C. Zeldin Division of Intramural Research, National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA

Part I
Volume 1

Structures of Cytochrome P450 Enzymes

1

Thomas L. Poulos and Eric F. Johnson

1.1 Introduction

The first cytochrome P450 structure, P450cam or CYP101A1, was solved in the early 1980s [1, 2], followed by the second, P450BM3, in 1993 [3]. At the time of the 3rd edition of this book published in 2004, there were a total of 13 unique P450 crystal structures deposited in the Protein Data Bank (PDB). As of April 2014, the PDB lists 449 entries with the name P450 in the title and of these about 54 are unique structures. The many new structures solved since the 3rd edition include various substrate/ligand complexes, P450s in various conformational states, and a few new P450-redox protein complexes. This wealth of new structural information has been particularly useful in a better understanding of P450 dynamics and how the P450 active site adapts to substrates of diverse sizes and shapes.

T. L. Poulos (✉)
Departments of Molecular Biology and Biochemistry, Pharmaceutical Sciences, and Chemistry, 2206 Natural Sciences 1, Mail Code 3900, University of California, Irvine, CA 92697-3900, USA
e-mail: poulos@uci.edu

E. F. Johnson
Department of Molecular and Experimental Medicine, The Scripps Research Institute, 10550N, Torrey Pines Rd., La Jolla, CA 92037-1000, USA
e-mail: johnson@scripps.edu

1.2 Overall Architecture

There now are a sufficient number of structures to safely state that the overall P450-fold is quite conservative. While it remains the case that there are no nonheme proteins that exhibit the P450-fold, there now are a small handful of examples of enzymes that exhibit the P450-fold but do not catalyze traditional P450 chemistry. These include the NO reductase, P450nor [4, 5], prostacyclin synthase [6–8], allene oxide synthase [8–11], P450BSβ [12], and a related peroxygenase, CYP152L1 [13], which hydroxylates fatty acids but does so using H_2O_2 as the oxidant.

The structures of six P450s are shown in Fig. 1.1, while Fig. 1.2 highlights some of the key secondary structural elements. Although the overall fold is maintained, the precise positioning of various structural elements differs substantially. In general, the closer to the heme, the more conserved the structure, especially helices I and L, which directly contact the heme. As expected, those regions controlling substrate specificity differ the most, especially the B′ helix. For example, in P450eryF, the B′ helix is oriented about 90° from the orientation observed in P450cam. The effect is a substantial change in local environment, which is required for substrate selectivity.

Not too surprisingly, the most conserved elements of the P450 structure center on the heme–thiolate oxygen activation chemistry. The most noteworthy is the β-bulge segment housing the Cys ligand (Fig. 1.3), just prior to the L helix. This rigid architecture is required to both protect

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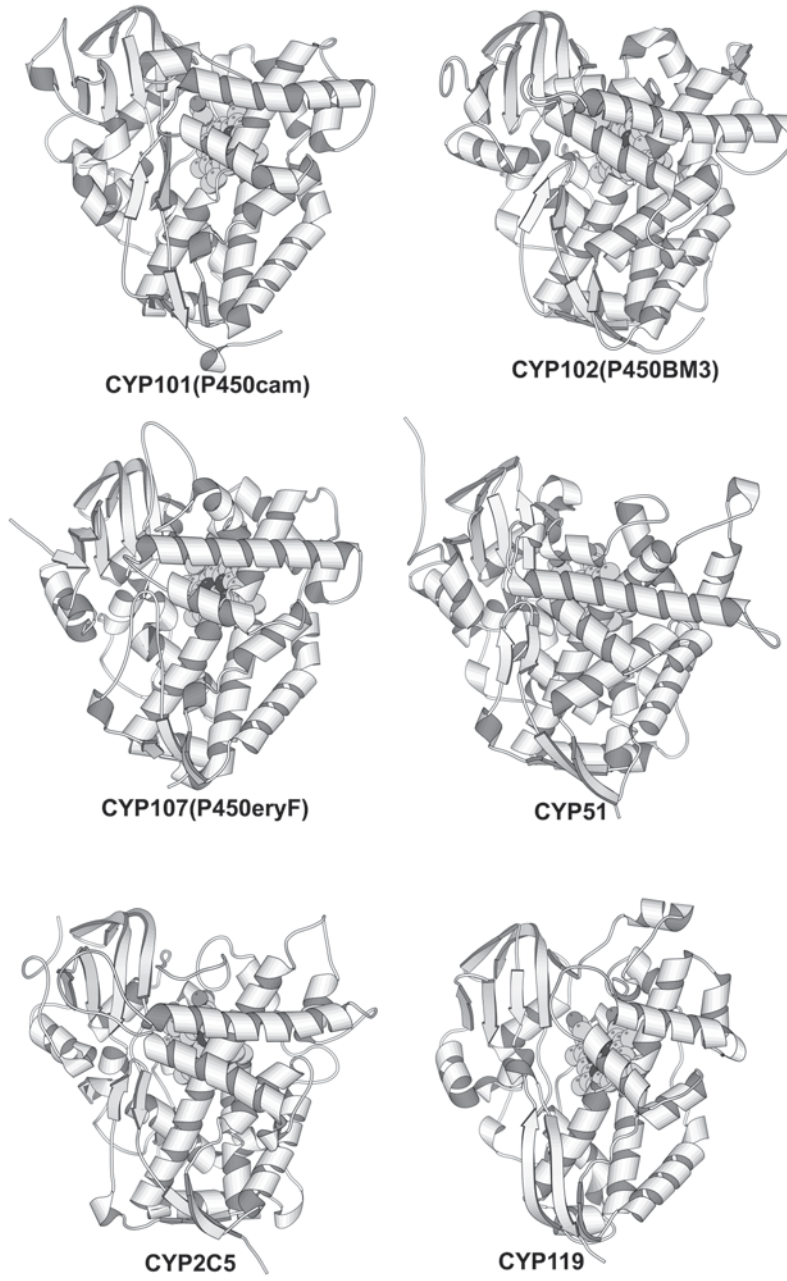

Fig. 1.1 A representative example of known P450 structures illustrating the common three-dimensional fold

that Cys ligand and hold it in place in order to be within H-bonding distances of two peptide NH groups, although the H-bonding geometry is good for only one H-bond. This arrangement is not only found in all P450s but also in two closely related enzymes, nitric oxide synthase (NOS) and chloroperoxidase (CPO). Both NOS and CPO are heme–thiolate enzymes that, like P450s, catalyze monooxygenation reactions. Like P450, the Cys ligand in CPO is near peptide bond NH groups [14]. NOS is similar, except that an H-bond is provided by the indole ring N atom of a

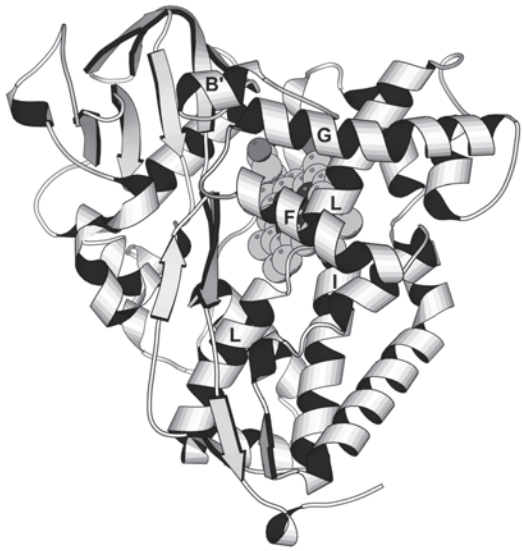

Fig. 1.2 The structure of P450cam (PDB: 5CP4) with key helical segments labeled. *PDB* Protein Data Bank

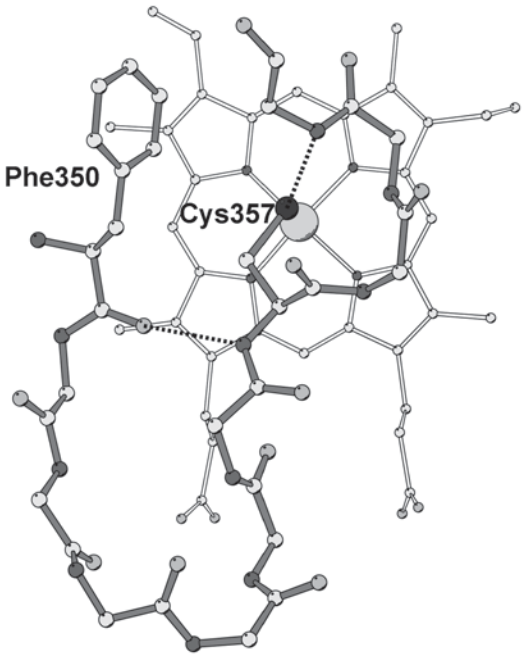

Fig. 1.3 The Cys ligand "loop" in P450cam. The *dashed lines* indicate key hydrogen bonding interactions that aid in stabilizing the Cys ligand. *Cys* cysteine

conserved Trp residue [15–17]. Such an H-bonding arrangement is not unique to heme–thiolate proteins, but is a characteristic feature of proteins containing Cys–Fe ligation, and was first observed in the ferredoxins [18]. These H-bonds aid in regulating the heme iron redox potential [19, 20]. Without such H-bonds, the redox potential would be too low for reduction by redox partners. Thus, it appears that the protein must provide a suitable electrostatic environment around the Cys ligand in order to maintain the redox potential in a physiologically accessible range. The same is true for a close cousin to P450, the peroxidases. Here histidine (His) serves as the axial ligand, but, in this case, it is necessary to increase rather than decrease the redox potential [21]. As a result, the His ligand H-bonds with a buried Asp residue that imparts greater imidazolate character to the His, thus lowering the heme iron redox potential [22–26].

The other highly conserved region involved in O_2 activation is the portion of helix I near the heme Fe (Fig. 1.4). Thr252 is involved in a local helical distortion in P450cam such that the threonine (Thr) side-chain OH donates an H-bond to a peptide carbonyl oxygen that would normally be involved in an α-helical H-bond. This Thr is not strictly conserved. For example, P450eryF contains an Ala instead of a Thr [27] and P450cin has an Asn [28]. Even so, these outliers also exhibit a similar distortion in the I helix. This arrangement is thought to be quite important for the proper delivery of protons to the iron-linked oxygen required for cleavage of the O–O bond, thus generating the active Fe–O hydroxylating species. The growing consensus is that ordered solvent at the active site serves as the direct proton donor to the iron-linked dioxygen [29–32]. P450–oxy complexes tend to be rather unstable, which is why there are only two crystal structures of P450–oxy complexes: P450cam [31, 33] and P450eryF [34]. In the P450cam–oxy complex, the I helix opens up slightly which provides sufficient room for two new waters to move into the active site. These waters form an H-bonded network that is thought to be important for the proper delivery of protons to dioxygen in order to promote heterolytic cleavage of the O–O bond (Fig. 1.4). While the positioning of new waters in the active site requires changes in the I helix, there are no changes in the P450eryF–oxy complex except

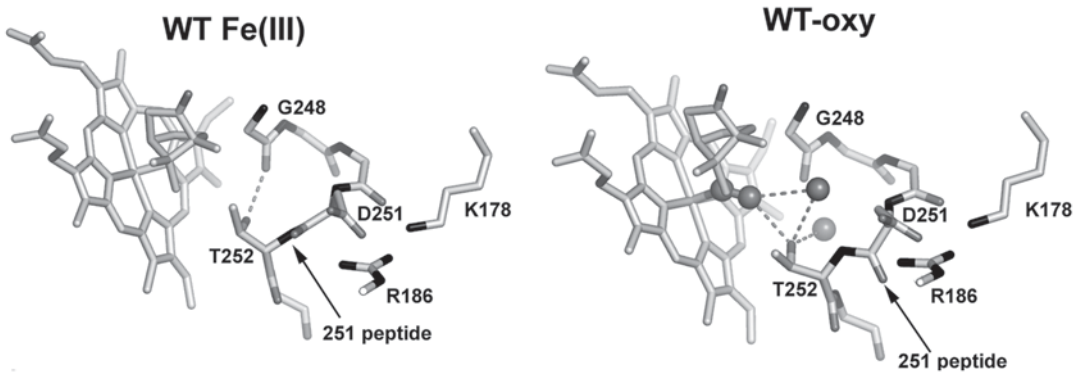

Fig. 1.4 A comparison of the I helix region in ferric and oxy-P450cam (PDB: 2A1M). When O_2 binds, the I helix opens up and the H-bond between Thr252 and Glu248 is broken. This opening enables additional waters to move into the active site that are thought to be critical for completing a protein relay network required for O_2 activation

for the repositioning of a water molecule. Since the conserved Thr252 found in P450cam is replaced by Ala in P450eryF (Fig. 1.5), the I helix is already in an open conformation similar to that of P450cam–oxy. It appears that P450eryF uses a substrate-assisted mechanism [35] since a substrate OH anchors the key water in place via H-bonding and is essential for activity. While the details of the proton shuttle machinery may differ from one P450 to the next, the surrounding protein groups and, in at least one case, the substrate, generally position solvent in the active site for proton delivery to dioxygen resulting in cleavage of the O–O bond.

1.3 Structural Features for Membrane Binding

In contrast to prokaryotic P450s, eukaryotic P450s are generally membrane-bound proteins. Most eukaryotic P450s are incorporated into the endoplasmic reticulum. However, several mammalian P450s that participate in the synthesis of

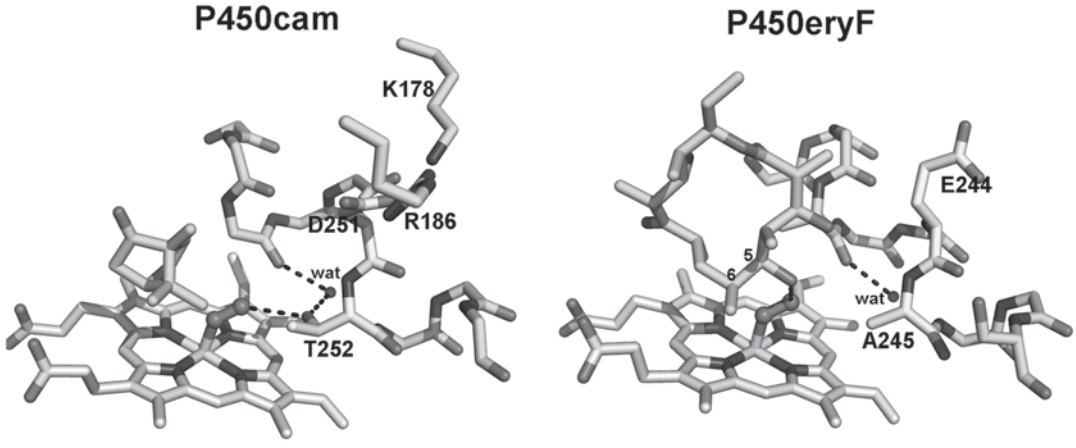

Fig. 1.5 A comparison of the solvent-mediated hydrogen bonding network in oxy-P450eryF (PDB: 1Z8O) and the oxy complex of P450cam. Unlike in P450cam, there is very little movement of the I helix in P450eryF when O_2 binds. This is probably because Thr252 in P450cam is replaced by Ala245 in P450eryF. As a result, the I helix is already more open in P450eryF

sterols, steroids, and bile acids are located on the matrix side of the mitochondrial inner membrane. A longer N-terminal polypeptide chain of roughly 30–50 amino acids precedes the catalytic domain in eukaryotic P450s and mediates membrane targeting. In the case of mitochondrial P450s, the targeting sequences are cleaved during import of the protein into the mitochondrion [36]. In contrast, the leader sequences of microsomal P450s are retained and inserted into the endoplasmic reticulum during protein synthesis [37]. The insertion process stops at the end of a hydrophobic stretch of roughly 20 amino acid residues, which are likely to form a helix in order to reduce the energetic costs of placing the polar peptide backbone in the nonpolar core of the bilayer [38]. A short linker region of about ten amino acids, which often includes positively charged amino acid residues, connects the transmembrane helix (TMH) to a generally conserved proline at the N-terminus of the structurally conserved P450-fold. The length of the 20 amino acid TMH corresponds roughly to the 3-nm width of the hydrocarbon core of the bilayer [39]. Additionally, the polar head groups of the phospholipids add another 1 nm outer layer on each side of the hydrophobic core, suggesting that a portion of the linker region resides in the polar head group layer.

The TMH is not required for function, as illustrated by the expression and successful reconstitution of several P450 monooxygenases in which this region was deleted [40–44]. Almost all of the currently available crystal structures have been determined for microsomal P450s expressed and crystallized without the TMH [45]. Initial structures of the human aromatase, CYP19A1, are an exception. Although the full-length aromatase was crystallized, the TMH and linker regions were disordered in the crystal [46]. Subsequently, engineered mutants of aromatase were expressed in *Escherichia coli* without the TMH, and these structures were not significantly affected by the absence of TMH [47].

Recently, additional evidence for the helical nature of the TMH was obtained from solid-state nuclear magnetic resonance (NMR) studies of rabbit microsomal CYP2B4 incorporated into magnetically oriented bicelles [48] and from a crystal structure reported for full-length, *Saccharomyces cerevisiae* CYP51A1, a sterol 14α-demethylase [49]. This crystal structure includes the linker region, TMH, and an additional amphipathic helix at the N-terminus. Interactions of the latter with a neighboring molecule in the crystal lattice contributed to a well-ordered N-terminus for structure determination. As a result, the predicted helical secondary structure of the TMH was confirmed, and a role for the additional amphipathic N-terminal helix in membrane binding to the distal leaflet of the bilayer was proposed, as illustrated in Fig. 1.6. Although the N-terminal amphipathic helix is not a general feature of microsomal P450s, this structure suggests that some P450s with extended N-terminal sequences could exhibit additional membrane interactions with the distal leaflet of the bilayer. In the *S. cerevisiae* CYP51A1 structure, the C-terminal end of the 24-residue TMH lies along the surface of the catalytic domain and passes from the proximal face to the distal face of the P450 along a trajectory that is roughly parallel with β-sheet 1, Fig. 1.6. The C-terminal end of the TMH helix corresponds to the linker region and is amphipathic with polar residues exhibiting hydrogen-bonding interactions with the catalytic domain and hydrophobic residues on the outer surface. This suggests that the observed trajectory is likely to be maintained when the enzyme is bound to the membrane [49]. As depicted in Fig. 1.6, a portion of the catalytic domain is likely to protrude into the membrane when the TMH resides in the lipid core. The surface of the catalytic domain surrounding this region is relatively hydrophobic for CYP51A1 (Fig. 1.7) as well as other membrane P450s [45, 50], which is likely to facilitate interactions with the lipid core of the bilayer.

This hydrophobic surface is formed by the N-terminal portion of the catalytic domain together with the helix F–G region, and there are distinctive structural differences between mammalian P450s and soluble prokaryotic P450s for this portion of the catalytic domain. The initial comparison of the first structure of a microsomal P450, CYP2C5, with structures of CYP102 and

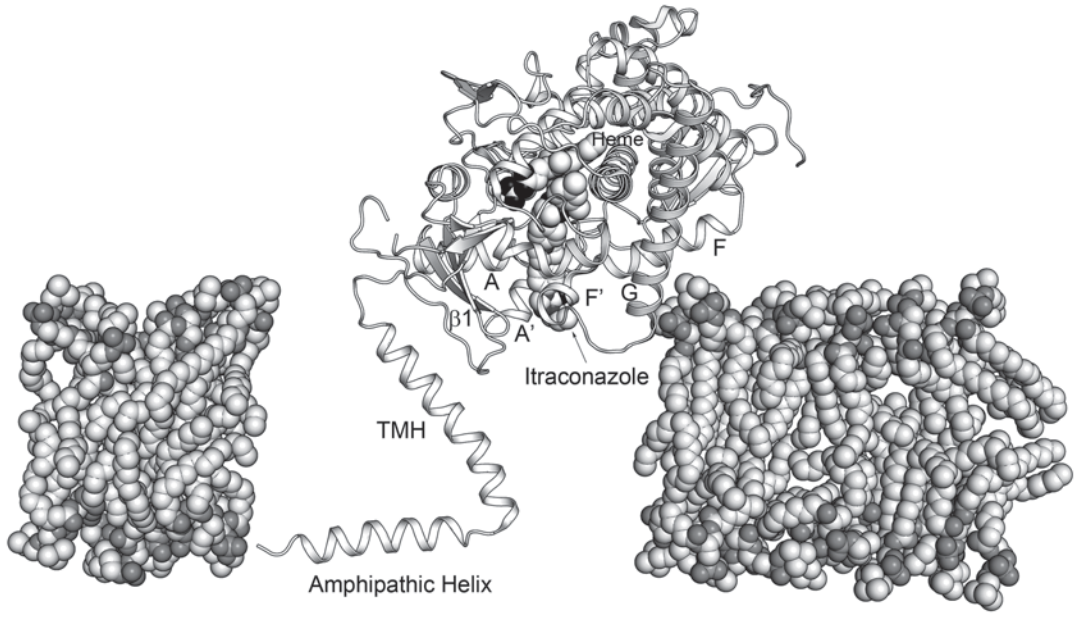

Fig. 1.6 Hypothetical model for the membrane binding of microsomal P450s. The cartoon depicts the experimentally determined fold of full-length, *Saccharomyces cerevisiae* CYP51A1 (PDB: 4KOF). For reference, the structure of the TMH is flanked by modeled arrays of phospholipid molecules depicted as CPK atoms. The amphipathic N-terminal helix is positioned at the transition of the polar head group and the hydrophobic layers with its hydrophobic surface oriented toward the lipid layer. The heme and bound inhibitor, itraconazole, are also rendered as CPK atoms. Itraconazole passes out of the access channel between helices A′ and F′, which are oriented toward the lipid portion of the bilayer. *TMH* transmembrane helix

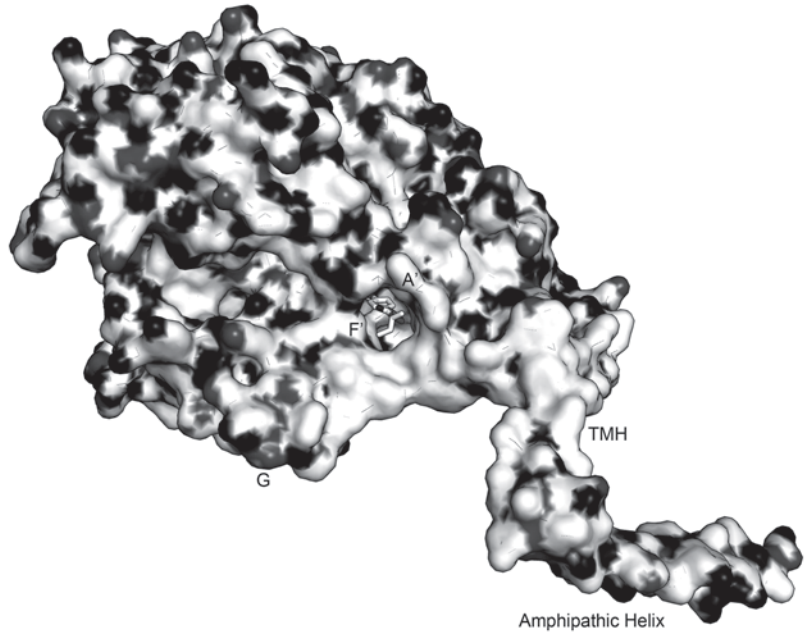

Fig. 1.7 Surface rendering of full-length, *Saccharomyces cerevisiae* CYP51A1, (PDB: 4KOF) with acidic and basic residues colored *black* and *gray,* respectively. Note the relative absence of charged residues on the surface of the catalytic domain surrounding the entrance channel and the TMH. Itraconazole is depicted as a stick figure in the entrance channel. The hydrophobic surface surrounding the entrance channel is oriented toward the membrane in Fig. 1.6. efj1

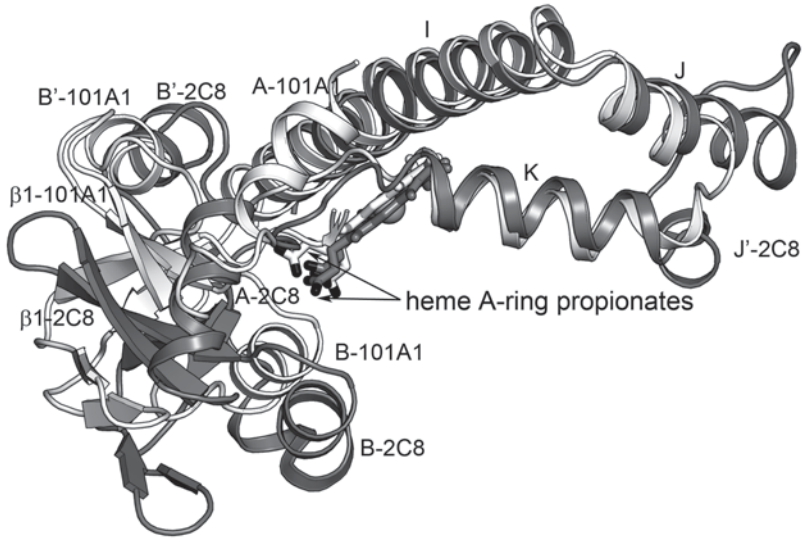

Fig. 1.8 Superposition of CYP101A1 (*light gray*) and CYP2C8 (*dark gray*). The hemes are shown as stick figures with oxygen atoms colored black. The heme iron is depicted by a *sphere*. Although helices *I* through *K* superimpose well, the N-terminal region is shifted outward for CYP2C8 relative CYP101. Different orientations are evident for the heme A-ring propionates

CYP101 [50, 51] indicated that the N-terminal portion of the catalytic domain from the linker region and to the helix B–C loop of CYP2C5 is shifted significantly toward the proximal face when compared to structures of prokaryotic P450s (Fig. 1.8). The generality of this observation was established by a retrospective analysis of a larger number of structures from diverse eukaryotic and soluble prokaryotic P450s by Denisov et al. [52]. As a result of this shift in position, the heme A-ring propionate is oriented toward the proximal side of the heme plane in most mammalian membrane P450s, where it often interacts with basic amino acid side chains. Notable exceptions are eukaryotic CYP51A1 and the non-monooxygenases, CYP8A1, a prostacyclin synthase and CYP74A1, a plant allene oxide synthetase. The heme A-ring propionate resides more typically on the distal side of the heme plane in prokaryotic P450s, with some exceptions. This shifted N-terminal/β-sheet domain resides near the connector between helices F and G, which is typically longer in eukaryotic P450s than in soluble, prokaryotic P450s. The structure of the F–G helical region varies extensively between mammalian P450s and often exhibits two short helices, F′ and G′. Together these elements form the hydrophobic surface near the N-terminus of the catalytic domain that is likely to be inserted into the membrane [45, 50].

The orientation of the hydrophobic surface toward the membrane is supported by studies indicating that antibody epitopes in this region are inaccessible to the antibody when CYP2B4 is in its native membrane [53], whereas epitopes on other portions of the molecule react with their respective antibodies. These and other epitope-mapping studies indicate that extensive portions of the surfaces of drug-metabolizing P450s are accessible to the antibodies when bound to membranes, as shown in Fig. 1.9 and reviewed in more detail [54]. Atomic force microscopy experiments estimate that the height of microsomal CYP2B4 above a model phospholipid membrane is roughly 35–45 nm [55]. This would require a portion of the protein to be buried in the membrane, which is likely to be the hydrophobic region near the N-terminus of the catalytic domain. Additionally, studies of the association of CYP2B4 with Langmuir–Blodgett phospholipid monolayers indicate that the protein displaces an area that is larger than a single TMH [56]. This

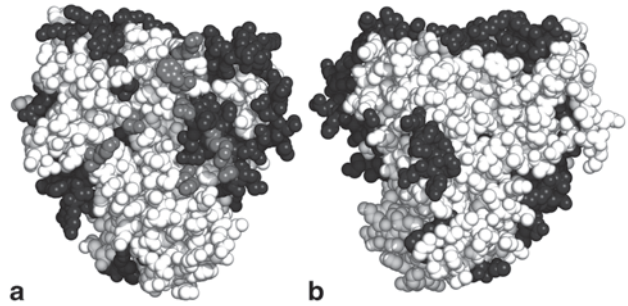

Fig. 1.9 CPK rendering of the proximal (**a**) and distal surfaces (**b**) of CYP2C5 (PDB: 1N6B). Antibody epitopes recognized when the P450s are bound to microsomal membranes are colored *dark gray,* as reviewed [54]. Several conserved amino acid side chains that have been implicated in P450 reductase interactions with CYP2B4 [156] are colored *medium gray*. The orientation of the protein is similar to that depicted in Fig. 1.6 efj-1 with the N-terminus of the catalytic domain positioned toward the *bottom* of the figure. (Reproduced from *Cytochrome P450, Third Edition* with permission from Springer Science+Business Media)

result would be consistent with the penetration of the hydrophobic surface of the protein into the adjacent leaflet of the lipid bilayer.

Molecular dynamics (MD) simulations of the binding of human microsomal P450s with phospholipid bilayers, reported initially for CYP2C9 [57–59] and CYP3A4 [52, 60], observed stable binding orientations for the catalytic domains with the hydrophobic surface of the catalytic domain immersed in the proximal leaflet of the phospholipid bilayer, Fig. 1.10. The structure of the catalytic domain was reported to be stable and exhibiting dynamic motion with root-mean-square deviation (RMSD) values of less than 2.5 Å from the starting structures.

The maximum heights of the catalytic domains above the membrane surface in these MD simulations are similar to that of 35–45 nm determined by atomic force microscopy for CYP2B4 [55]. Additionally, the tilt of the heme plane relative to the membrane normal, Fig. 1.10, in the models of membrane-binding interactions can be compared to results from biophysical studies for this angle. This tilt angle has been estimated for CYP17A1 and CYP21A2 based on the anisotropic decay of the absorption spectrum following photodissociation of carbon monoxide complexes by polarized light. This approach gives two solutions for the angle of the orientation of the heme plane relative to the membrane normal of either 43° or 27° and 52° or 12°, respectively for the two enzymes [61]. The larger values are similar to a single value for tilt angle of 59.7 ± 4.1° estimated from the dichroic ratio observed for the absorption of visible light by the heme chromophore of P450 3A4 bound to nanodisc membranes. Tilt angles for the heme in the initial MD simulations for P450 2C9 were reported to be 55 ± 5° [58], and in additional MD simulations for P450s 1A2, 2A6, 2C9, 2D6, 2E1, and 3A4, using similar conditions, the heme-tilt angles differed between P450s and ranged from 56 ± 5° for CYP3A4 to 72 ± 6° for CYP2D6 [59]. Differences between P450s are not unexpected, as the distal surfaces of microsomal P450s differ significantly, and these differences are likely to affect the angle tilt and extent of membrane insertion.

Heme-tilt angles observed for CYP3A4 in MD simulations from two different studies were reported to be 68.7°–75.9° [60] and 56 ± 5° [59]. The reported differences between the two MD simulations could reflect differences in the model membranes used in the simulations, as well as different initial models for the N-terminus used in the MD simulations. As structures for the native N-terminal domains were not available for CYP3A4, and the other proteins characterized in these studies, they were modeled de novo with the hydrophobic portion of the N-terminus modeled as a TMH. The structure of the linker region in these proteins is less certain, and is likely to vary between P450s. X-ray crystal structures of

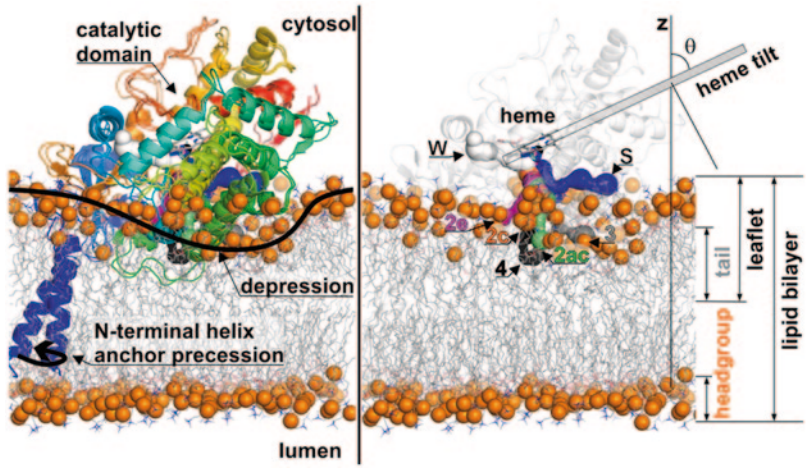

Fig. 1.10 Immersion of CYP2C9 in a dioleoylphosphatidylcholine (DOPC) lipid bilayer. (*Left*) Overlaid snapshots of CYP2C9 taken at 0.1 and 1 μs molecular dynamics (MD) simulations showing that the catalytic domain is immersed in a membrane depression framed by lipid phosphate groups (shown as *orange spheres*). Water molecules are not shown for clarity. The N-terminal helix shows precessional movement about the bilayer normal. The fold of the catalytic domain is conserved and agrees with that observed in X-ray crystallography experiments. (*Right*) Snapshot taken at 1 μs of MD simulation showing positions of active site access and egress channels computed from the heme moiety using MOLE 2.0.20. The water channel (*white*) points toward the cytosolic environment, whereas solvent channel S (*blue*) points above the lipid head groups. All other channels point inside the bilayer. Channels 2e, 2c, and 3 point into the lipid head group region, whereas channels 4 and 2ac point below the lipid head groups. The heme tilt angle θ (between the heme plane and the bilayer normal z, i.e., defined according to Baylon et al. [60] is depicted. (Reprinted with permission from [59], copyright 2013 American Chemical Society. The channels are designated as described [93])

mammalian microsomal P450s have generally been determined for proteins without their N-terminal TMH, and, in most cases, the native linker regions of family 2 P450s were modified to correspond to the linker region of CYP2C3, as described for CYP2C5, [40, 41, 50]. Moreover, the structures of these short N-terminal regions have not been defined for many P450s. CYP3A4 is an exception [62, 63], and the native linker region exhibits an A″ helix following a turn that directs the polypeptide chain along β-sheet 1 from the N-terminus of the catalytic domain near the hydrophilic proximal face toward the hydrophobic distal surface. This trajectory is similar, but not identical, to that observed more recently for the structure of full-length *S. cerevisiae* CYP51A1, Fig. 1.6. The initial model used by Baylon et al. [60] incorporated a flexible link between helix A″ and the TMH, which provides some flexibility for the orientation of the TMH independently of the catalytic domain during the MD simulation. The initial model used by Berka et al. [59] for the N-terminus CYP3A4 was based on their earlier equilibrated CYP2C9 model obtained following a 0.25 μs MD simulation [58]. Interestingly, the helix A″ region and the TMH of the initial model of P450 2C9 were built as a continuous helix, but a kink developed between helix A″ region and the TMH during the MD simulation that allowed the polar Arg side chains in the linker region to reside in the polar region of the bilayer, and the TMH to span the lipid core of the membrane, as illustrated in Fig. 1.10 by a 1 μs equilibrated model from a later study [59]. Helix A″ may not be a generally conserved feature for linker regions, as the same segment of the native linker regions does not exhibit an A″ helix in the structure of human CYP1A2 [64], and is not evident in the MD simulation model of CYP1A2 [59]. Both CYP1A2 and CYP3A4 exhibit short N-terminal helices that are roughly orthogonal to the TMH and that are positioned at the interface between

the polar head group and lipid layers of the distal leaflet of the bilayer in the MD simulations of Berka et al. [59]. These models are similar to the bimodal membrane binding proposed for full-length *S. cerevisiae* CYP51A1 [49].

Although the MD simulations generally support the notion that a portion of the distal surface is embedded in the membrane surface, the results of biophysical experiments and topology studies often show differences that are difficult to reconcile with a single model. Fluorescent quenching of tryptophan residues introduced on the surface of CYP2C2 by site-directed mutagenesis suggested that residues 36 and 69 flanking helix A and 380 in β-sheet 2 of CYP2C2 are inserted into the fatty acyl core of the bilayer, while residue 80 on helix B and 225 at the turn between helices F′ and G′ are in the polar region of the phospholipid bilayer [65], leading the authors to propose a more vertical orientation for CYP2C2 than was observed in the MD simulations for the closely related CYP2C9 [57, 58]. Experimental evidence indicates that P450s are present as both monomers and dimers in membranes [66, 67], and a more vertical orientation relative to the membrane surface would be consistent with models for the dimerization of the catalytic domain of N-terminally truncated P450 2C8 in aqueous solution that involve interactions of the helix F–G loop region [63]. This model for the dimerization of 2C8 is supported by cross-linking studies for the membrane-bound full-length CYP2C8 [68]. Additionally, these cross-linking studies implicated the linker region and TMH in the dimerization of membrane-bound, full-length CYP2C8 expressed in mammalian cells or in *E. coli* membranes. Cys-scanning mutagenesis indicated that reactive cysteines reside on a single side of the TMH, whereas several consecutive residues were reactive in the linker region suggestive of a more flexible structure. This flexibility is necessary for reorientation of the proximal faces relative to TMH in order to form a P450 dimer through interactions of the helix F–G region. P450 dimerization in membranes is thought, in some cases, to inhibit reduction by the microsomal cytochrome P450 reductase, so the monomer is likely to be the predominant functional form of the enzyme [66].

As mitochondrial P450s lack the N-terminal TMHs found in microsomal P450s, the interactions of the catalytic domain with the matrix side of the inner membrane are likely to be the predominant membrane interaction. Consistent with a role for the helix A′, F′, and G′ regions in membrane binding, these regions exhibit nonpolar, exterior surfaces in structures of mitochondrial P450s 11A1 [69, 70], 11B1 [71], and 24A1 [72]. Moreover, substitutions of more polar residues for hydrophobic residues on the F′ and G′ surfaces increase salt extractability and solubility of mitochondrial P450 27A1 [73]. The helix F–G region of mitochondrial P450 11A1 is also protected from chemical modification by membrane association [74]. Similarly, microsomal P450s expressed without their TMH retain capacities to bind to phospholipid membranes, and mutations made to the helix F′ and G′ regions of microsomal P450s 2C5 [41], 2D6 [75], and 7A1 [76] facilitate extraction in high salt buffers. These observations suggest the extended loop between helices F and G in eukaryotic P450s contributes to membrane binding for both mitochondrial and microsomal P450s.

1.4 Conformational Dynamics for Substrate Access

Many P450 structures are in the so-called closed state with no obvious way that substrates can gain access to the active site. As a result, substrate entry and product egress may involve rather large conformational changes. Once the P450cam structure became available, an immediate puzzle was how camphor gains access to the active site since the substrate is buried, and there is no obvious opening. The substrate-free and bound structures showed no differences, although substrate-free P450cam exhibited higher thermal motion in the B′, F, and G helices, suggesting that these regions must move to allow substrate to enter the active site [77]. The first clear indication that conformational changes are important in substrate binding was the struc-

ture of palmitoleic acid bound to P450BM3 [78], which was followed by a higher-resolution structure [79]. Interestingly, the experimentally observed conformational change was correctly predicted based on computational methods [80, 81] before the substrate-bound crystal structure was solved. The main motion involves the F and G helices sliding over the surface of the I helix. This motion closes off the entry channel, indicating that substrates enter near the F/G loop region that is similar to that of P450cam.

There now are a handful of P450 structures in the open and closed forms and in all of them, the F and G helices and the F/G loop undergo large changes. Not surprisingly, the most extensive analysis has been with P450cam. In the open form [82], the F and G helices move, and the B′ helix region becomes disordered. It also has been possible to trap the P450cam access channel using a series of tethered compounds where the substrate is attached to a long linker that extends out of the active site [83, 84]. A principal component analysis of 30 different tethered compound structures indicates that there are three dominant conformational states available to P450cam: closed, partially open, and fully open [84].

Two close homologues to P450cam with about 46% sequence identity with P450cam, CYP101D1 [85] and CYP101D2 [86], have now been characterized. Both catalyze exactly the same reaction as P450cam, but there are substantial differences with respect to the open and closed states and the relationship between spin-state and substrate binding. For example, CYP101D2 has been crystallized only in the open state, but camphor can be soaked into the crystals and binds in the active site [86]. The camphor, however, does not bind in a productive mode, but instead the carbonyl O atom of the substrate H-bonds with the water coordinated to the heme iron (Fig. 1.11). MD simulations of CYP101D2 show that this P450 can adopt various conformational states, mainly by motions of the F/G helical substrate access channel, and provides a dynamic picture of substrate binding consistent with other P450s [87]. Perhaps the most unexpected difference between P450cam and its close cousins is that camphor binding to CYP101D1 gives only about 40% high spin even with excess substrate. In addition, the Fe_2S_2 ferredoxin that supports CYP101D1 catalysis, Arx, is able to reduce substrate-free 100% low-spin CYP101D1, while only high-spin substrate-bound P450cam can be reduced by its redox partner, Pdx. In addition, Pdx can support CYP101D1 catalysis, while only Pdx can support P450cam catalysis [88]. There is nothing obvious in the structures that can explain these differences other than the fact that in CYP101D2 the substrate can bind to the low-spin open state, albeit in a nonproductive binding mode (Fig. 1.11). One simple way of rationalizing these differences is to hypothesize that CYP101D1 can bind camphor in various orientations that are consistent with a water molecule remaining coordinated to the heme iron, as in CYP101D2, thus giving a substrate-bound mostly low-spin complex. Upon reduction of the heme iron, the water ligand is displaced and the substrate can "relax" to a productive binding mode. This hypothesis requires that CYP101D1 is "looser" than P450cam and can more readily adopt the open conformation. The static X-ray structures do not reveal anything obvious to support this scenario, and proof one way or the other must await other approaches more in tune with measuring dynamic differences.

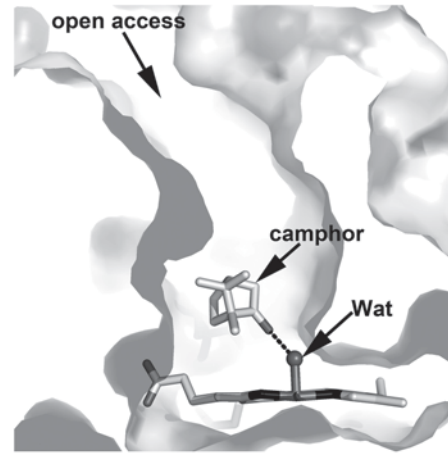

Fig. 1.11 The open substrate-binding channel in CYP101D2 (PDB: 3NV6) [86]. The substrate camphor binds but is not oriented in the productive binding mode. Instead, the camphor carbonyl O atom H-bonds with the water coordinated to the iron

1.5 Substrate Access to Membrane P450s

Similar to prokaryotic P450s, membrane P450s have been crystallized in both open and closed conformations. For example, rabbit microsomal CYP2B4 and human CYP2B6 have been crystallized in closed forms, as illustrated in Fig. 1.12, by a CYP2B6 4-(4-chlorophenyl)-imidazole complex [89] and in open forms, as illustrated by a complex of CYP2B6 with one molecule of amlodipine coordinated to the heme iron and a second molecule bound in the entry channel and protruding between helix F′ and A′ [90]. These two conformations of CYP2B6 differ in the positions of the helices A′, A, B′, F, F′, and G. Open forms of rabbit CYP2B4 have also been determined where the helix F′–G′ and helix B–C regions are displaced to a much greater extent by ligand and detergent interactions [91, 92]. Mammalian drug-metabolizing enzymes such as CYP2B4 bind a wide-range of compounds, and conformational changes are often associated with the capacities of these enzymes to facilitate the metabolic clearance of many compounds by accommodating large compounds in an open access channel [45]. Mitochondrial P450s also exhibit open and closed structures. CYP11A1 and CYP11B1 exhibit closed structures for substrate complexes with the helix F and F′ region blocking the substrate access channel described earlier for CYP101, Fig. 1.13. In contrast, mitochondrial CYP24A1 was crystallized in an open conformation with a large cleft between helices A′ and helices F′–G′. As discussed in the previous section, these helices are likely to bind to the membrane, and the hydrophobic substrates cholesterol and vitamin D_3, respectively, could enter each enzyme from the membrane. Most P450 substrates exhibit partition coefficients that favor the hydrophobic environment of the bilayer over the aqueous phase, which suggests that the concentration of substrate in the membrane may be higher than in solution under physiological conditions.

Structures obtained with bound ligands are often closed, and substrate access channels remain closed during MD simulations that are of short duration compared to substrate dissociation rates. Nevertheless, a number of solvent channels have been identified in X-ray crystal structures and during MD simulations in an aqueous medium for soluble and truncated membrane P450s

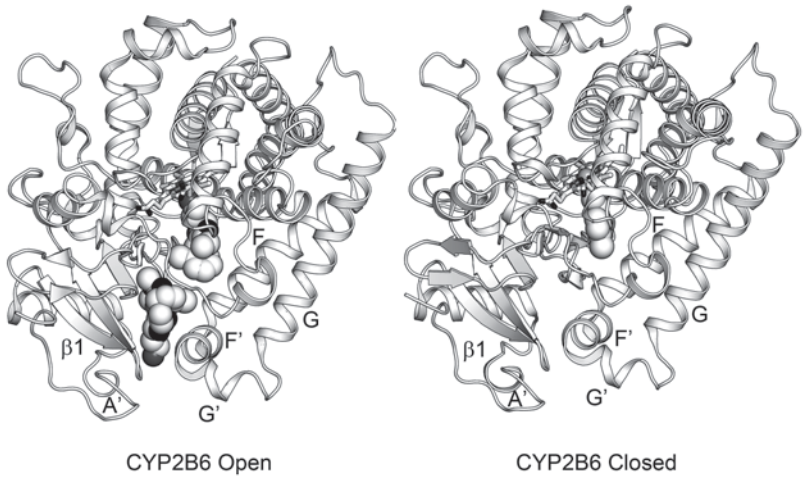

Fig. 1.12 Open (PDB: 3UA5) and closed (PDB: 3IBD) conformations of human CYP2B6. The open structure has two molecules of amlodipine (*spheres*) with one molecule of amlodipine bound to the heme iron via nitrogen coordination and the second amlodipine in the open-substrate access channel. The closed structure has one molecule of 4-(4-chlorophenyl)imidazole (*spheres*) coordinated to the heme iron with a closed substrate entrance channel. The heme is rendered as a stick figure with the iron shown as a sphere. Nitrogen and oxygen atoms are colored *light gray* and *black,* respectively

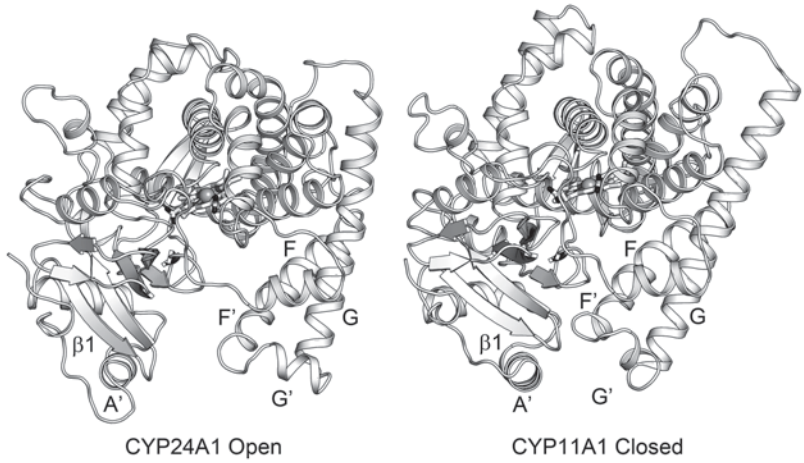

Fig. 1.13 Open (PDB: 3KNV) and closed (PDB: 3NAO) conformations of mitochondrial rat CYP24A1 and bovine CYP11A1, respectively. The heme is rendered as a stick figure with the iron shown as a *sphere*. Nitrogen and oxygen atoms are colored *light gray* and *black,* respectively

[93]. As shown in Fig. 1.10, several of these channels are oriented into the lipid portion of the bilayer in MD simulations. Comparisons of the duration and extent of opening during MD simulations, for the catalytic domains in an aqueous environment and bound to membranes, are qualitatively similar and reveal differences in the frequency and duration of channel opening that reflect interactions between the catalytic domain and the membrane [57–60]. These solvent channels are thought to open and coalesce to form substrate access channels as seen for open conformations of soluble and membrane P450s determined by X-ray crystallography [93].

1.6 Substrate Complexes: Specific P450s

A fascinating structural feature of P450s is the ability to adapt to substrates of various sizes and shapes, yet retain the overall P450-fold and P450 electron transfer and O_2 activation chemistries. Most of our detailed understanding of protein–substrate interactions derives from highly specific P450s that bind their respective substrates tightly and thus generate crystals that diffract well. Several substrates for various specific P450s are shown in Fig. 1.14. The size and shape of the various substrates shown in Fig. 1.14 are sufficiently diverse that the structural basis for what controls substrate specificity can, at least in part, be understood. As expected, all substrates are situated such that the atom to be hydroxylated is within 4–5 Å of the heme iron. Thus, regio- and stereoselective hydroxylation by the Fe(IV)-O species is achieved by specific protein–substrate interactions that hold the substrate in the correct position. The exception is P450BM3. The structure of the P450BM3 heme domain with palmitoleic acid [78] and N-palmitoylglycine [79] show that the fatty acid substrate is ≈7–8 Å from the iron which is too far for hydroxylation. However, NMR results indicate that the substrate moves to be within 3 Å of the iron upon reduction from Fe(III) to Fe(II) [94]. Precisely how reduction is linked to such a large repositioning of the substrate remains unknown.

P450cam and P450epoK [95] represent the two extremes of substrate size and shape. Hence, a comparison between these two structures provides some insights on which regions of the structure change most in response to the requirements of substrate specificity. The two regions that differ the most between P450epoK and P450cam are the F, G, B′ helices, and the F/G loop (Fig. 1.15). The B′ helix is rotated 90° in P450epoK compared to P450cam. This re-

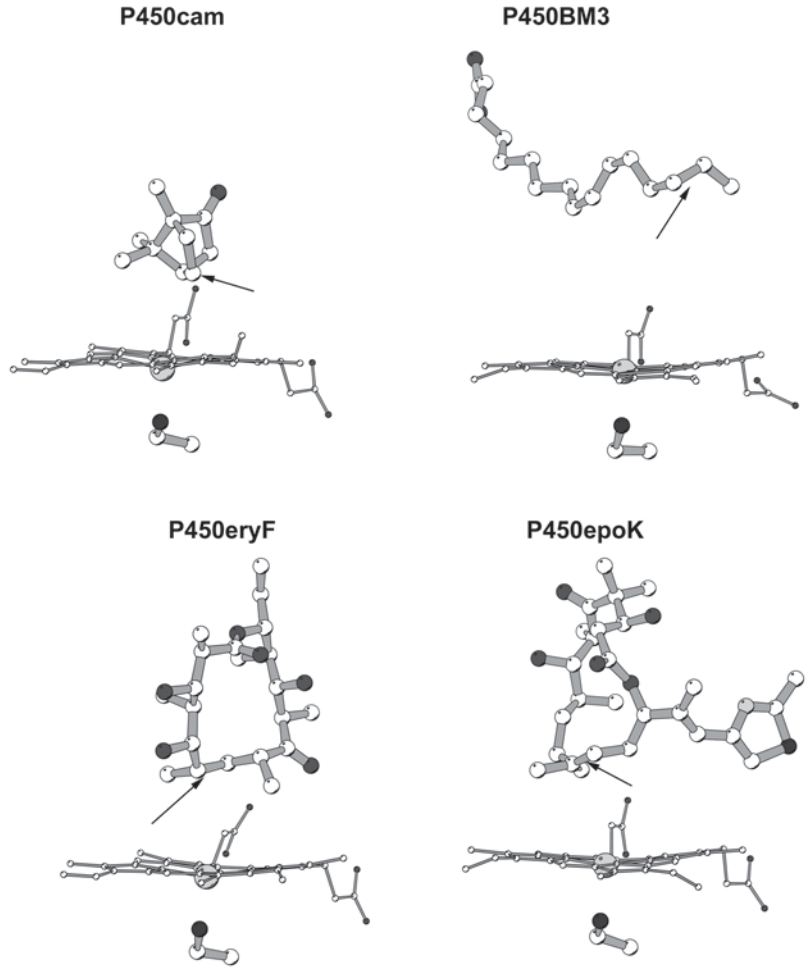

Fig. 1.14 Substrates bound to the active site of various P450s

orientation opens the substrate-binding pocket, thus making room for the thiazole ring of the substrate. The F and G helices do not superimpose well, and the F/G loop adopts a substantially different conformation. There also are examples where a second substrate molecule is trapped in the access channel possibly because crystallization favors a partially open active site, thus leaving room for an additional molecule. Anecdotal observations not usually published show that *E. coli* "mystery" molecules will sometimes bind in the access channel or active site. This likely reflects the general hydrophobic nature of P450 active sites and the open/close dynamics that might make it possible for even specific P450s to bind different molecules present in the growth media.

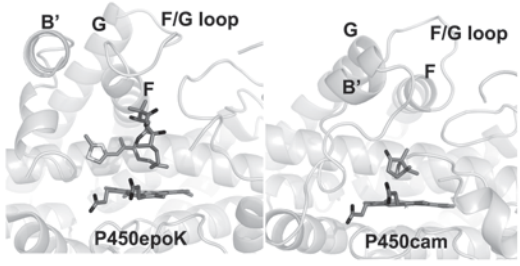

Fig. 1.15 A comparison of the P450cam and P450epoK (PDB: 1PKF) active sites. The very different size and shape of the substrates illustrate how the active site substantially differs from one P450 to the next

An unusual example of a P450–substrate interaction is CYP107H1 (P450BioI). P450s participate in polyketide biosynthesis, and these

pathways involve multiple enzymatic steps that process a growing fatty acid-like chain into the array of complex and well-known antibiotics and other natural products. In many of these systems, an acyl carrier protein (ACP) forms a covalent bond with the substrate and transfers the substrate from one enzyme to the next. Where hydroxylation reactions are required, P450s often are involved, which means that in some of these systems the substrate is delivered to the P450 by the carrier protein. One well-characterized system is from the biotin biosynthetic pathway in *B. subtilis* [96]. P450BioI catalyzes the formation of pimelic acid through the oxidative cleavage of a fatty acid carbon–carbon bond, which then proceeds on to biotin [97, 98]. There is now a crystal structure of such fatty acid acylated ACP protein complexed with the P450 (Fig. 1.16) [99]. Structurally, P450BioI is a typical P450, yet here the substrate entry pocket has been adapted to bind ACP. Note that the substrate enters the active site near the connection between the F and G helices that is the main entry point for substrates in many P450s.

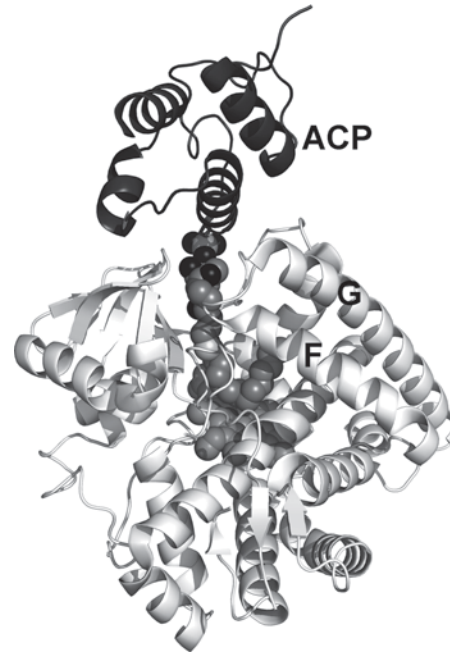

Fig. 1.16 The crystal structure of P450BioI (PDB: 3EJB) [99]. ACP (*darker* molecule) binds such that the fatty acid substrate attached to ACP extends into the active site of the P450. The opening near the F/G loop region that enables substrate entry is the same as observed in many other P450s. *ACP* acyl carrier protein

There is one final example of P450 substrate adaptability, but in this case there may be two different active sites and two enzyme activities. CYP170A1 from *Streptomyces coelicolor* catalyzes the oxidation of epi-isozizaene to an epimeric mix of 5-albaflavenol (Fig. 1.17). The structure shows that there are two substrate molecules bound, one in the expected location just above the heme and a second in the substrate access channel [100]. What was most unexpected is the finding that the conversion of farnesyl diphosphate to epi-isozizaene is catalyzed by CYP170A1. Sequence comparisons between known sesquiterpene synthase enzymes pointed toward a particular region of CYP170A1 that might be involved (arrow in Fig. 1.17). Subsequent mutagenesis in this region eliminated the synthase activity but not the P450 activity [100]. Given that we are accustomed to viewing enzymes as requiring a relatively large size to properly form the active site, it might at first seem odd that such a small region of a P450, or any enzyme, could serve a catalytic function. However, sesquiterpene synthase enzymes appear not to operate by typical acid–base catalysis requiring suitably positioned active site groups to move protons [101]. Instead, it appears that metal ions and the substrate diphosphate are the keys to catalysis and that the enzyme may serve a more passive role, providing a template for substrate and metal ion binding.

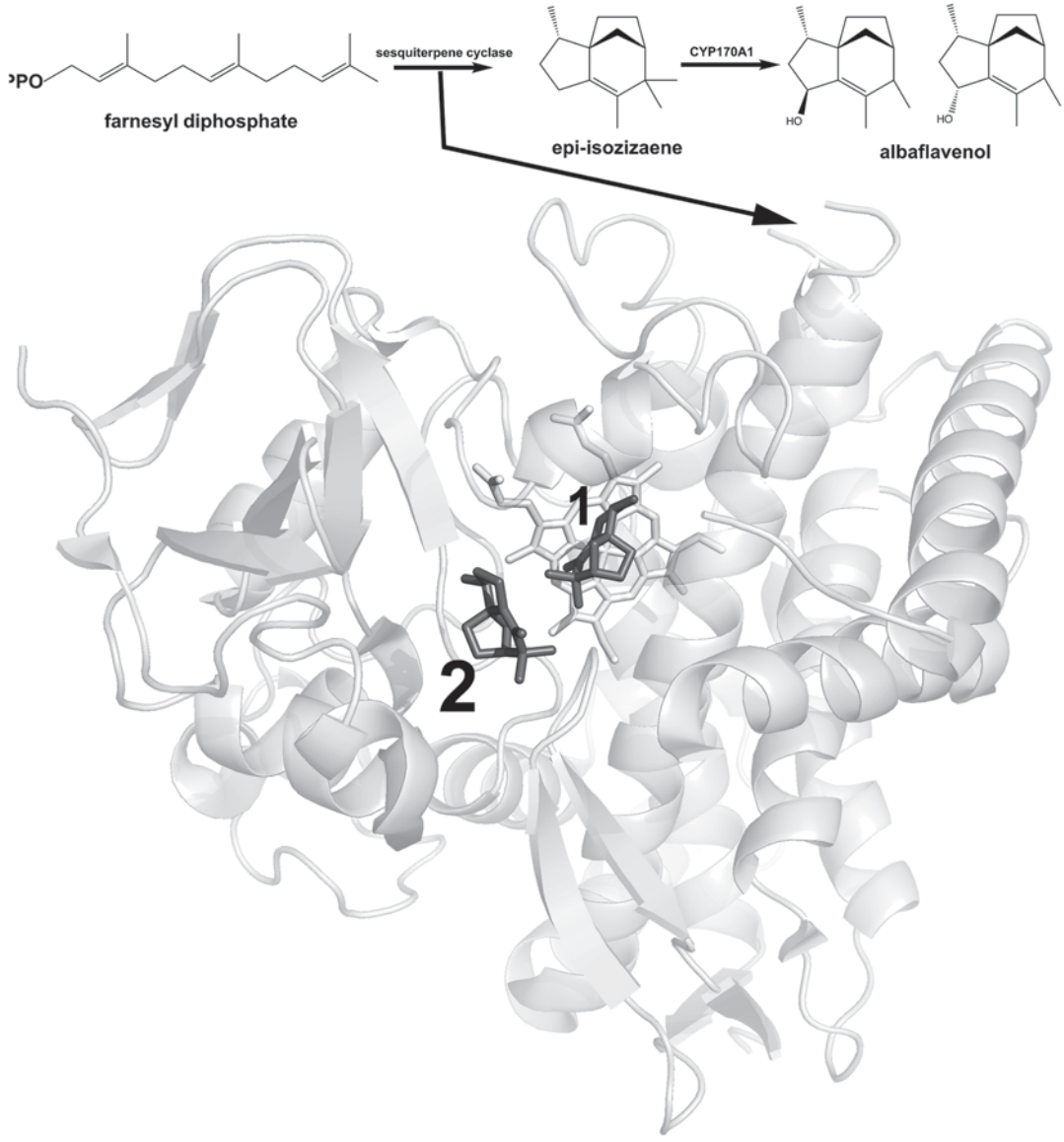

Fig. 1.17 The CYP170A1 (PDB: 3DBG) crystal structure [100] and reaction. Substrate *1* binds near the heme as expected, while substrate molecule *2* binds in the open access channel. The site thought to be responsible for the sesquiterpene cyclase activity is indicated by the *arrow*

1.7 Active Site Diversity of Mammalian P450s

As with prokaryotic P450s, active site diversity underlies the unique roles of P450s in mammalian physiology. Structures now are available for several of the enzymes that hydroxylate the aliphatic side chains of cholesterol and vitamin D_3. P450 11A1 catalyzes three successive oxygenation reactions to produce sequentially 22*R*-hydroxycholesterol, 22*R*,20*R*-dihydroxycholesterol, and an unstable product that undergoes carbon–carbon bond scission to produce the 21-carbon steroid, pregnenolone, and isocaproaldehyde. It is thought that the peroxyanion intermediate that precedes formation of the oxene is the reactive intermediate for the third reaction [102]. The crystal structure of human mitochon-

drial CYP11A1 with cholesterol bound [70] indicates that the tetracyclic sterol moiety is bound in the entrance channel to the substrate-binding cavity under the helix F–F′ region and above β-sheet 1 with C22 of the aliphatic side chain positioned closest to the heme iron, Fig. 1.18a. Additional structures of P450 11A1 [69, 70] with the first and second products of the reaction, 22*R*-

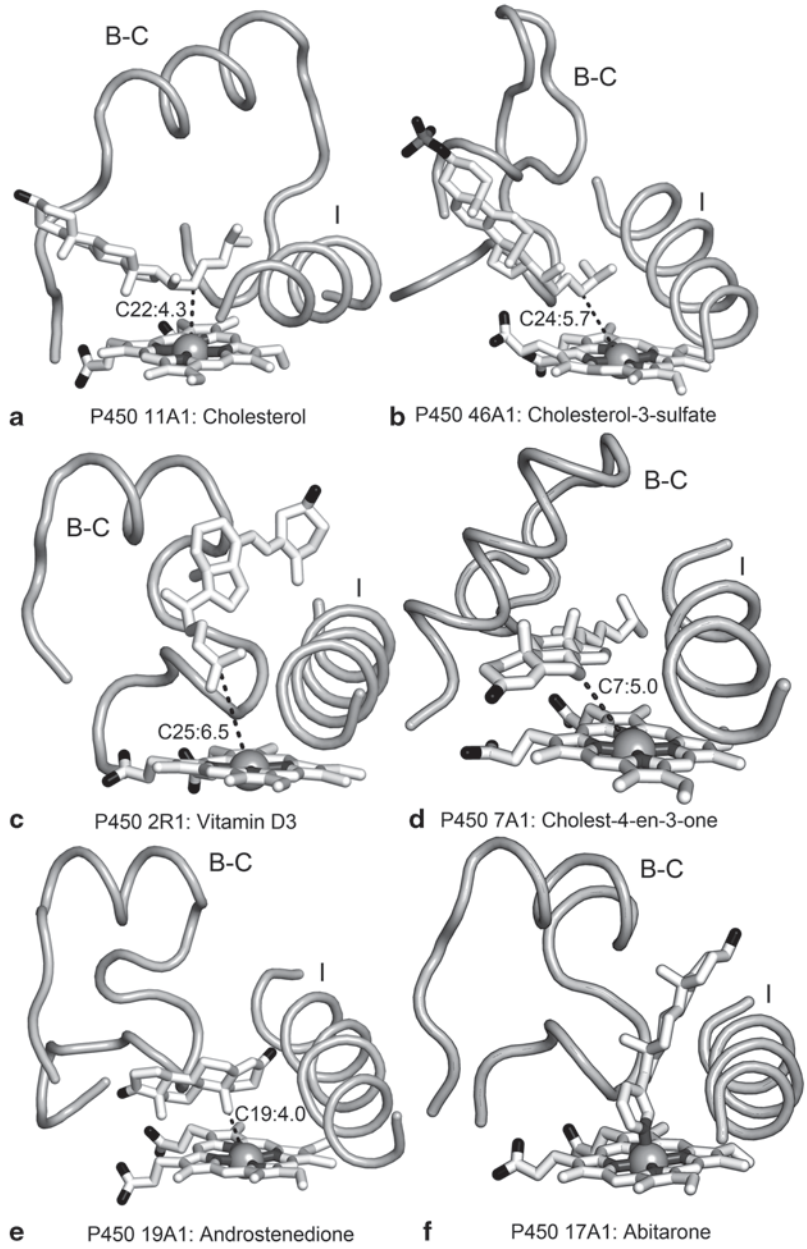

Fig. 1.18 Substrate and inhibitor binding to human P450s that catalyze key steps in steroid metabolism. The substrates, inhibitor, and heme are shown as stick figures with the heme iron depicted as a *sphere*. Nitrogen and oxygen atoms are colored *light gray* and *black,* respectively. The *dotted lines* represent the distance from the heme iron for sites of metabolism labeled with the identity of the site of metabolism and the distance. The CYP17A1 inhibitor abiraterone binds directly to the heme iron. For reference, a portion of helix I and the helix B–C loop are shown. The topology and length of the helix B–C loops exhibit significant variation between proteins

hydroxycholesterol and 22*R*,20*R*-dihydroxycholesterol, indicate that the tetracyclic sterol moiety is positioned similarly to that of cholesterol in each case, with changes in the dihedral angles of the side chain positioning the appropriate site of metabolism close to the heme iron. The structure of CYP46A1 [103] indicates that cholesterol sulfate binds similarly, but the aliphatic side chain is positioned with C24 closest to the heme iron, Fig. 1.18b. In contrast, an X-ray crystal structure of P450 2R1 [104] reveals that the sterol moiety of vitamin D_3 is located under helix G near helices I and B′ with the site of metabolism, C25, positioned closest to the heme iron, Fig. 1.18c.

Other steroid biosynthetic enzymes catalyze reactions that modify the rigid tetracyclic steroid ring system. Three enzymes, P450s 7A1, 7B1, and 39A1, insert an oxygen atom into the 7α C–H bond to produce 7α-hydroxylated intermediates in the formation of bile acids. A structure of P450 7A1 with the cholesterol analog, cholest-4-en-3-one (PDB code *3SN5*), indicates that the 7α C–H bond is positioned closest to the heme iron and that the plane of the sterol ring is parallel to the plane of the heme, Fig. 1.18d. The aliphatic side–chain passes out of the substrate-binding cavity between helix I and the helix B′–C loop. Structures of P450s 19A1 [105] and 11B1 [71] also place the tetracyclic steroid ring system of androst-4-ene-3,20-dione and 21-hydroxyprogesterone in a similar location, but with the C19 methyl group, Fig. 1.18e, and the 11β C–H bond, respectively, oriented toward the heme iron. P450 19A1 catalyzes three successive oxidations of the 19-methyl group with the product rearranging to produce formic acid and the unsaturated A ring of the estrogen, estrone.

Other reactions catalyzed by steroid biosynthetic enzymes target the ends of the steroid ring system. The structure of the adrenal 21-hydroxylase [106] with 17α-hydroxyprogesterone bound reveals that the tetracyclic steroid is oriented almost perpendicular to the plane of the heme with the 17β-side chain positioned near the heme iron. P450 17A1, which catalyzes the 17α-hydroxylation of progesterone and cleavage of the 17β-side chain of the pregnenolone to form androstenedione, has been crystallized with abiraterone [107] in the active site, Fig. 1.18f. Abiraterone is used clinically for the treatment of prostate cancer via inhibition of androgen formation catalyzed by P450 17A1. The steroid moiety of abiraterone is oriented similarly to 17α-hydroxyprogesterone in the P450 21A2 structure, with abiraterone coordinated to the heme iron through a heterocyclic nitrogen group. Structures of human CYP51A1 with inhibitors bound in the active site are also available to aid in the development of CYP51A1 inhibitors that will target these enzymes in pathogens without inhibiting the human enzyme [108, 109].

As is evident in Fig. 1.18, P450's have evolved to catalyze these reactions by positioning the substrates for site-selective metabolism, and, in doing so, different portions of the P450 structure are utilized for substrate binding. This, in turn, reflects differences in the sizes and properties of the amino acids that occupy the active site cavity as well as changes in protein conformation. Examples of these conformational differences are readily apparent when comparing the helix B–C loop regions depicted in the six panels of Fig. 1.18.

In contrast, P450s in families 1A, 2A, 2B, 2C, 2D, 2E, 2J, and 3A frequently contribute to the metabolic clearance of drugs and other xenobiotics. In the absence of evolutionary selection to optimize the binding of these compounds, many xenobiotic substrates are likely to exhibit relatively poor fits in P450 active sites and several isoenergetic binding poses may be possible, as suggested by the formation of multiple metabolites. Reaction rates are likely to reflect probabilities for binding to specific enzymes, relative reactivity of potential sites of reaction, and probabilities for placement of the sites of reaction near the oxene intermediate, leading to uncoupling, multiple metabolites and poor catalytic efficiencies. Fortunately, the enzymes that catalyze these reactions exhibit significant active site diversity that provides protection from a wide range of structurally diverse xenobiotics.

Family 1 and 3 enzymes exhibit very different active-site cavities. The enzymes in family 1 typically metabolize polynuclear aromatic hydrocarbons, and the structures of human CYP1A1 [110], 1A2 [64], and 1B1 [111] exhibit narrow active-site cavities that complement the size and

planarity of polynuclear aromatic hydrocarbons, as illustrated for CYP1A2 in Fig. 1.19a. These narrow active-site cavities are reinforced by a kink in helix F, which directs a portion of helix F and helix F′ between the active site and the N-terminal domain. This is likely to add rigidity to the narrow active site cavity. In contrast, CYP3A4 exhibits a large and open active site cavity (Fig. 1.19b) with a much larger exposure of the heme surface to substrates than seen in other xenobiotic metabolizing enzymes [62, 112]. This difference underlies the capacity of 3A4 to catalyze oxygenation of the steroids at carbons 6 or 7 in the center of the ring system as seen for steroidogenic CYP7A1 in Fig. 1.18. CYP3A4 is also unusual because helix F is short and does not cross above the active site. As a result, the active site can expand and contract by the flexible motion of the long connector between helix F and F′ and changes in the positions of helices F′ and G′ [113]. The active-site cavities of human family 2 P450s range from small for P450s 2E1 [114, 115], 2A6 [116] Fig. 1.19c, 2A13 [117], and 2B6 [89, 90] to large for 2C8 [63], Fig. 1.2C9 [118, 119], 2C19 [120], and 2D6 [75, 121], and they can vary due to conformational changes associated with ligand access and binding [45]. As such, these enzymes contribute diverse capacities for xenobiotic metabolism.

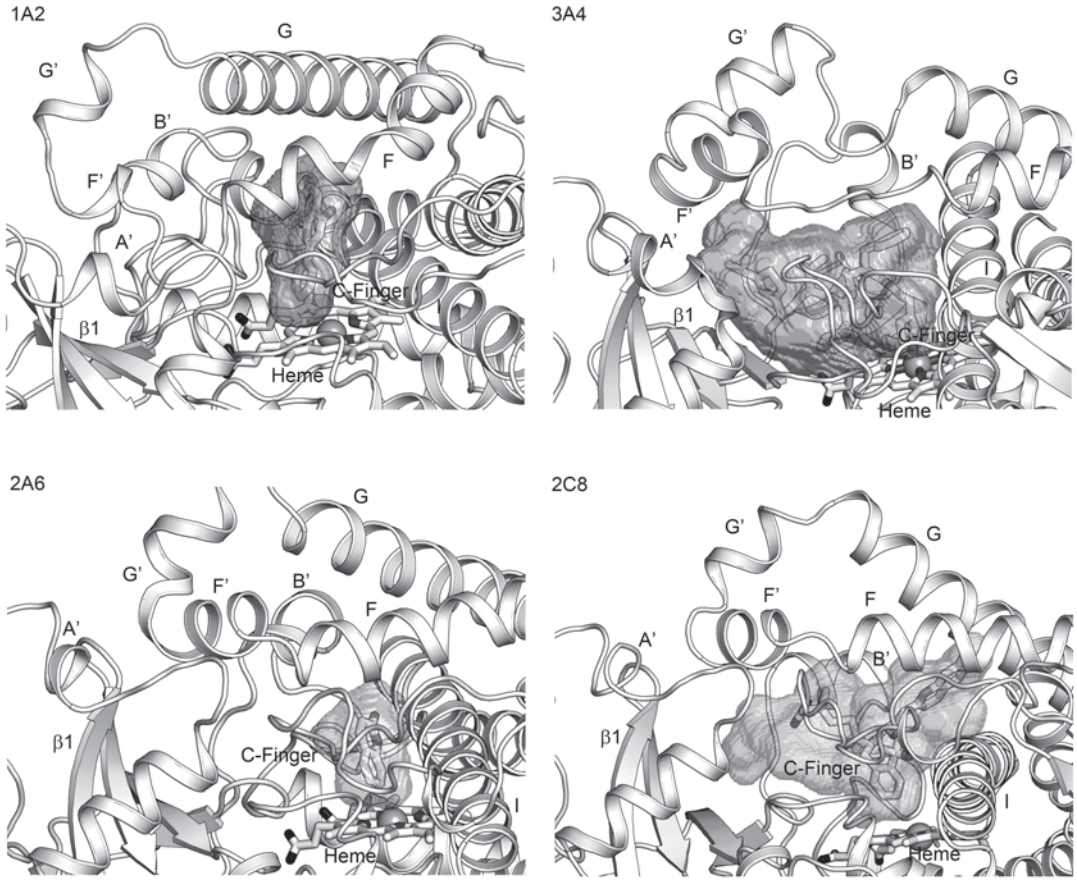

Fig. 1.19 Portions of the structures of the complex of CYP1A2 with α-naphthoflavone (PDB:2HI4), CYP3A4 with ritonavir (PDB:3NXU), CYP2A6 with coumarin (PDB:1Z10), and CYP2C8 with montelukast (PDB:2NNI) are shown as cartoons displaying secondary structures. The heme and ligands are depicted as stick figures with the heme iron shown as a *sphere*. The surfaces of the active-site cavities were calculated using VOIDOO [157] and rendered as a transparent surface. Nitrogen and oxygen atoms are colored *light gray* and *black,* respectively

1.8 Electron Transfer Complexes

P450s do not operate alone but must form a complex with a redox partner for electron transfer. Protein redox complexes, including those involving P450s, are designed not to be very tight or long-lived. A complex that is too tight will have a slow dissociation rate, which precludes rapid turnover. Nature thus must strike a balance between specificity, affinity, and high turnover. Such complexes have proven quite difficult to crystallize, which is why there are very few protein–protein redox complexes in the PDB and, to date, there are only three crystal structures of a P450 complexed with a redox partner.

The first structure of a redox complex to be solved was that between the heme and FMN domains of P450BM3. Although P450BM3 is a bacterial enzyme, P450BM3 is more closely related in sequence, structure, activity, and redox partner to microsomal P450s than to other bacterial P450s. The unique feature of P450BM3 is that the diflavin P450 reductase is linked to the C-terminal end of the heme domain, thus giving a catalytically self-sufficient enzyme. Crystals were obtained by removing the FAD domain [122]. The structure (Fig. 1.20) shows that the FMN domain docks on the proximal surface of the P450, which was expected, based on complementary electrostatic surfaces and mutagenesis studies. The linker connecting the heme and FMN domains had been proteolyzed during crystallization, thus raising the possibility that the structure is an artifact of crystallization. Further experiments were carried out to test the functional validity of the model. Residues found at the interface were probed by mutagenesis [123]. Replacing Leu104 of P450BM3 with a Cys (Fig. 1.20) at the interface should not alter binding or electron transfer because replacing Leu with a smaller side chain should not cause any steric problems in forming the proper complex. However, covalent modification of the mutant Cys104 side chain with a large fluorophore should interfere with electron transfer. For these studies, laser flash photolysis was used wherein a laser flash photoreduced a potent reductant, deazariboflavin, which in turn reduces the FMN in the complex. The reduced FMN semiquinone then reduces the P450 heme. As predicted, mutation of Leu104 to Cys had no effect, while chemical modification of Cys104 dramatically decreased the FMN-to-heme electron transfer rate, thus

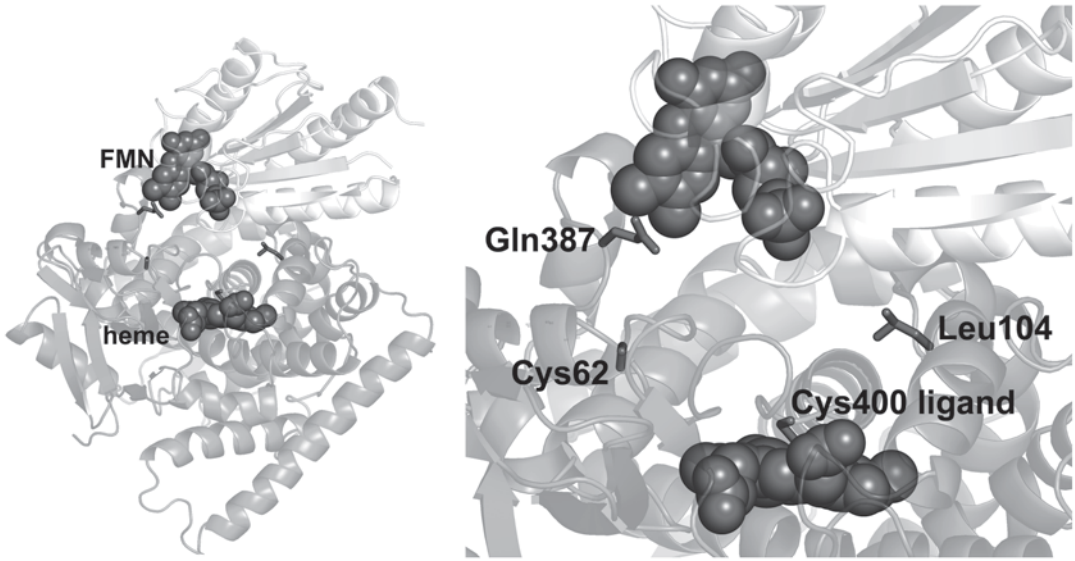

Fig. 1.20 Crystal structure of the P450BM3 electron-transfer complex (PDB: 1BVY) [122]. The closest contact at the interface is between Gln387 in the heme domain and the FMN

implicating Leu104 as an important residue in forming the proper electron transfer complex.

A second prediction from the P450BM3 electron-transfer complex structure that can be tested is the electron transfer path. The heme–FMN domain interface is shown in Fig. 1.20. The closest point of contact between the two domains places the FMN about 4 Å from the peptide backbone of Gln387. The peptide chain from Gln387 to the heme ligand, Cys400, could constitute an electron transfer path. To test this hypothesis, Gln387 was converted to Cys and modified with (4-bromomethyl-4′-methylbipyridine) [bis(bipyridine)]ruthenium(II) [124]. The covalently attached Ru(II) is photoreduced, and the rate of reduction of the heme Fe(III) to Fe(II) by the photo-generated Ru(I) was followed. The same experiment was carried out with Ru(II) attached to Cys62. Both Cys62 and Cys387 are about the same distance from the heme, but electron transfer from Cys60-Ru(II) must make "through-space" jumps, while there is a continuous covalent connection between Cys386-Ru(II) and the heme ligand, Cys400. In the case of Cys387-Ru(II), the heme iron was reduced at a rate of $4.6 \times 10^5\ s^{-1}$, while Cys60-Ru(II) did not reduce the heme iron. These results indicate that if the crystal structure of P450BM3 electron-transfer complex is functionally relevant, then the electron-transfer reaction can readily proceed along the direct point of contact between the FMN and heme domain.

The structure of the complex formed between adrenodoxin (Adx) and P45011A1, which converts cholesterol to pregnenolone, also has been solved. The crystal structure of the complex was solved by fusing adrenodoxin to the N-terminal end of CYP11A1 [70]. Although a good part of the Adx was disordered and not visible in electron-density maps, the interface with CYP11A1 was well defined (Fig. 1.21). The interface is dominated by electrostatic interactions and those residues involved are consistent with mutagenesis and chemical modifications studies [125–127]. A comparison between the free enzyme [69] and the enzyme complexed with Adx are essentially identical, so Adx binding does not result in any significant structural change.

The most recent structure to be determined is the P450cam–Pdx complex crystal [128, 129] and NMR structures [128]. The P450cam–Pdx complex has received considerable attention es-

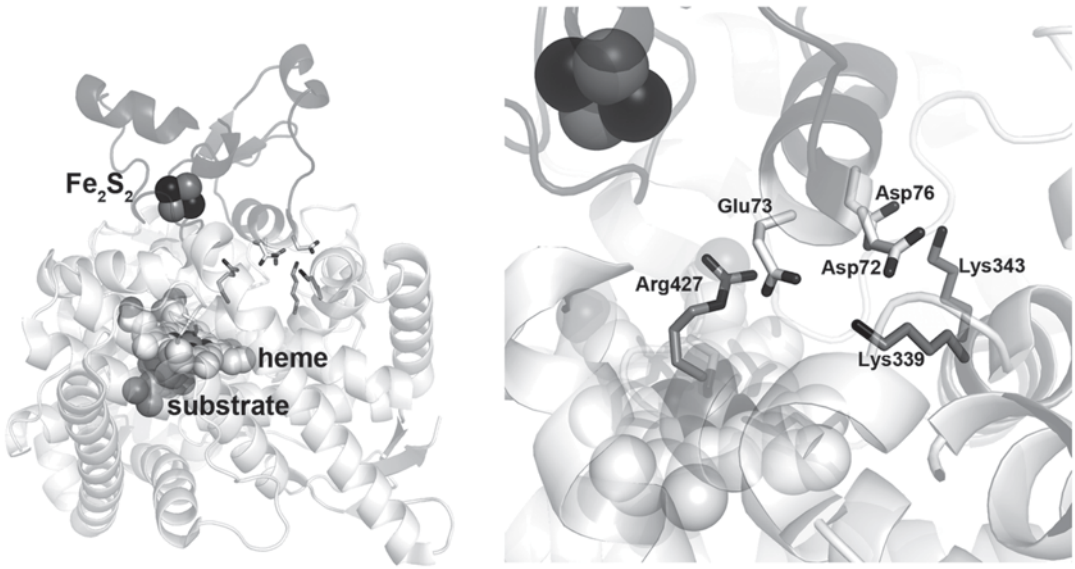

Fig. 1.21 Crystal structure of the complex formed between CYP11A1 and adrenodoxin (Adx; PDB: 3N9Y) [70]. Only part of the Adx is visible in electron-density maps. The interface is dominated by ionic interactions. Adx binding does not result in any major structural change in CYP11A1

pecially since it was established some time ago that P450cam is not only very selective for Pdx but Pdx also plays an effector role by inducing structural changes required for electron transfer and O_2 activation [130–132]. Prior to the recent crystal structure of the P450cam–Pdx complex, Pochapsky et al. developed a model of the P450cam–Pdx complex using NMR and molecular modeling [133] that is supported by mutagenesis data [134–138]. A wealth of spectral data shows that when Pdx binds on the proximal side of the heme, spectral changes ensue that are associated with the opposite distal substrate-binding pocket. These changes include resonance Raman [139], infrared [138, 140], and NMR [141–143]. NMR studies [144–146] showed that Pdx binding results in changes in the B′, C, F, and G helices that are well removed from where Pdx binds (Fig. 1.22). The B′ helix provides key contacts with the substrate, while large movements of the F and G helices are the main features of the open/close transition [82]. Pdx binding to oxy-P450cam decreases the stability of the oxy complex 150-fold [147], while oxidized Pdx shifts oxidized P450cam to the low-spin state [148]. All these observations point to significant structural changes in P450cam when Pdx binds.

The crystal structure of the P450–Pdx complex [128, 129] shows that P450cam adopts the open conformation, which is consistent with previous spectroscopic studies. The structure of the reduced form of the complex has four P450–Pdx molecules in the asymmetric unit and in three of these, the product, hydroxycamphor, is bound [129]. This means that the open form in the complex is active in O_2 activation and hydroxylation. Interactions at the interface are consistent with earlier NMR studies [145] and mutagenesis data [43, 137, 138, 149–153]. Pdx_{Asp38} interacts with $P450cam_{Arg112}$ (Fig. 1.22), which requires little movement in either protein in the vicinity of the ion pair. However, interactions involving Pdx_{Trp106}, which has been known for some time to be a critical residue [131], require movement of the C helix (Fig. 1.22). In effect, the C helix moves "up" about 2–3 Å in order to form nonpolar and H-bonding interactions with Pdx_{Trp106}. This motion of the C helix is coupled to movements in the B′, I, F, and G helices, all of which are involved with substrate access or direct contacts with both substrates, camphor and O_2. This motion results in a large movement of the F and G helices and the F/G loop, which effectively opens the active site to bulk solvent. This open conformation is

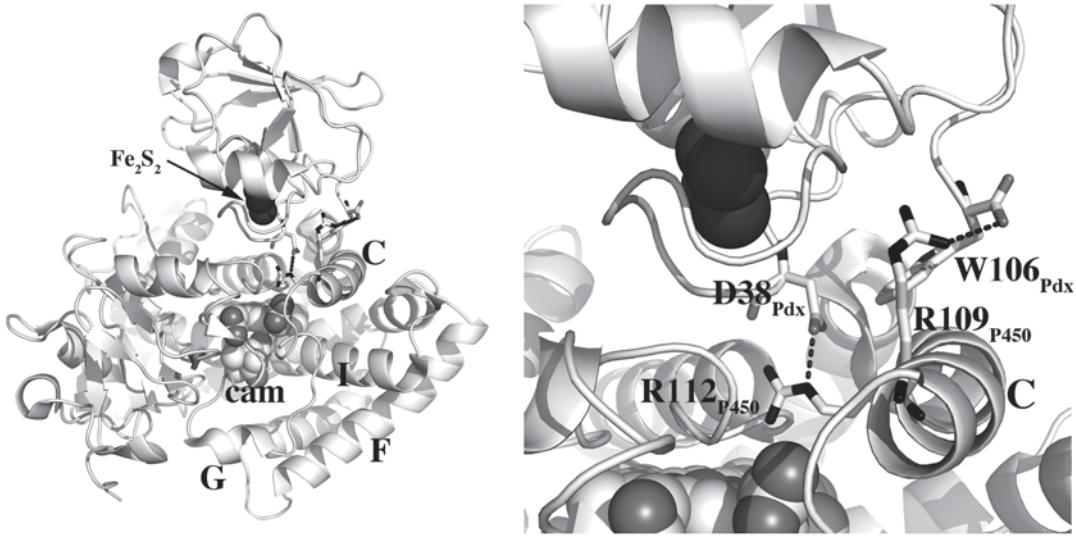

Fig. 1.22 Structure of the P450cam–Pdx complex (PDB: 4JX1) [129]. A key interaction is between Pdx_{Trp106} and the C helix in P450cam. The C helix moves "up" in order to optimize interactions with Pdx_{Trp106}. This motion is coupled to an opening of the active site access channel on the opposite side of the protein (F/G helical region)

the same as observed by Lee et al. [82] The main difference is that in the structure solved by Lee et al. [82] the B′ helix is disordered, while in a complex with Pdx the entire P450cam is highly ordered and the key interactions between camphor and the local environment remain, by and large, unchanged from the closed conformation. The main driving force for the conformational change appears to be Pdx_{Trp106}, which could not form tight interactions with P450cam without the structural switch.

The central question is why such Pdx-induced changes are important for activity. A possibly important part of the Pdx-induced structural change centers on the I helix near the O_2-binding site. The switch in the I helix in going from the closed to open state results in opening of the I helix similar to what happens when O_2 binds (Fig. 1.5). The closed to oxy-complex opening of the I helix is about midway between the extremes of the closed to fully open switch. This opening of the I helix is required to enable the catalytic waters to move into place for proton transfer to dioxygen [31, 33]. Thus, Pdx binding helps to stabilize the more oxy-like conformation of the I helix. However, the oxy-P450cam structure probably does not represent the final active state since Pdx binding perturbs the oxy-P450cam spectrum and results in a 150-fold destabilization of the oxy complex [147]. It has been argued that Pdx "pushes" the oxy complex more toward the active form that is probably the more open conformation. Pdx also alters the electronic properties of the thiolate ligand [147, 148], which could be due to a shortening of the peptide NH-thiolate H-bond observed in the P450cam–Pdx crystal structure.

Another large change that occurs when Pdx binds involves Asp251. Asp251 is part of the I helix and is usually Asp or Glu in many other P450s. Asp251 is essential for activity in P450cam [154], P450cin [155], and CYP101D1 [88]. The Asp251Asn mutant in P450cam exhibits a two-orders-of-magnitude decrease in activity, yet remains tightly coupled [154]. That is, nearly all the electrons funneled into the P450cam mutant are utilized for substrate hydroxylation and not the wasteful production of water or peroxide. This mutant also exhibits a kinetic solvent isotope effect of 10 compared to 1.8 for wild-type P450cam [32]. This strongly implicates Asp251 as being intimately involved with the proper delivery of protons to dioxygen required for heterolytic cleavage of the O–O bond. The problem with this view, however, is that Asp251 is tied up with Arg187 and Lys178 in two strong ion pairs (Fig. 1.23). However, in the Pdx complex,

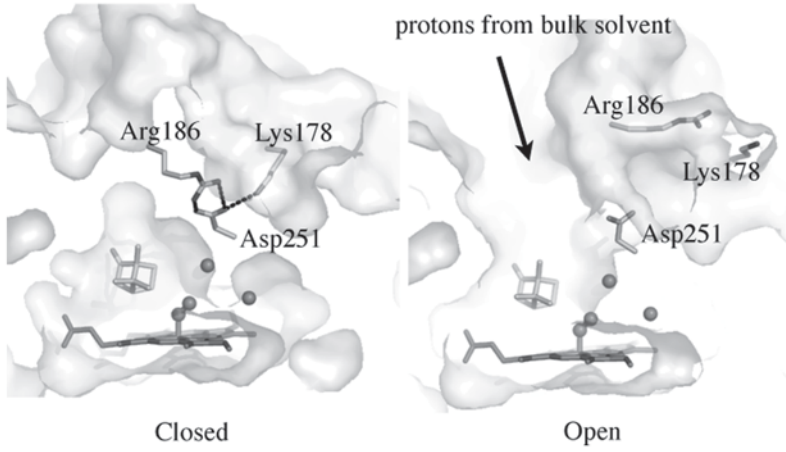

Fig. 1.23 The region around Asp251 in P450cam with and without Pdx bound. In the Pdx-free closed state, Asp251 is tied up in strong ion pairs with Arg186 and Lys178. When Pdx binds these ionic interactions are broken, thereby releasing Asp251 for its role in shuttling solvent protons to the iron-linked O_2 molecule required for O–O bond cleavage and thus, O_2 activation

these ion pairs are broken, which frees Asp251 to serve its proposed role in shuttling protons from bulk solvent into the active site. It thus appears that an important part of Pdx binding may be to "arm" the proton delivery machinery required for proton-coupled electron transfer.

The next obvious question is whether or not this sort of redox partner-mediated conformational change required for activity is a general property of all P450s or is limited to P450cam. The weight of the evidence so far indicates that P450cam may be an outlier. A number of P450s are known to be supported by nonphysiological redox partners and some redox partners, such as P450 reductase, service a large number of P450s. The only structural comparisons that can be made to address this question are the P450cam–Pdx and CYP11A1–Adx complexes [69]. CYP11A1 does not change to the open form in the complex but remains closed [69, 70]. However, Asp290 (corresponds to Asp251 in P450cam) is not tied up in ion pairs and is exposed to bulk solvent. Hence, no structural changes are required to free Asp290 for catalysis, although it has yet to be established if Asp290 is essential for CYP11A1 catalysis. Given that Nature has so many P450s, it is doubtful that P450cam is the only P450 where selective redox partner binding coupled with conformational selection is required for activity. It should only be a matter of time before similar P450s are uncovered and analyzed in depth. Just as interesting a question is the biological basis for such control. What is the evolutionary advantage, if any, of P450cam exhibiting such specificity, while very closely related P450s do not?

1.9 Conclusion

The large increase in P450 crystal structures over the past few years is due in large part to technological advances in protein expression and purification. Just as important are the increasing genome databases which now makes it relatively easy to "discover" new P450s. The sophistication of user-friendly crystallization robots, software, and synchrotron data collection has opened up crystallography to the nonexpert which also has been a major contributor to the ever-expanding number of structures deposited in the protein database. In fact, the field is now at the stage where expression, purification, characterization, and crystal structure determination can outpace functional and biological studies. Many structures now are being solved before one knows much about function. We thus must start using structural information to guide functional and biological studies. This could be particularly important with orphan P450s that will continue to increase in number as more and more P450s are discovered in new and interesting places. Such advances coupled with powerful computational resources that can be used for molecular modeling and in silico screening of potential substrates can significantly contribute to a better understanding of function. Recent advances in defining various conformational states also is quite important since which conformational state one uses for virtual screening of substrate/inhibitors is obviously quite important. Now, however, we have a better idea on the various conformational states available to P450s which will further sharpen predictive computational tools. We thus anticipate that P450 structural biology will continue to move quickly but that much less time and energy will be devoted to the actual structure determination and instead, will be focused on function.

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Electron Transfer Partners of Cytochrome P450

2

Lucy Waskell and Jung-Ja P. Kim

Abbreviations

POR	NADPH-cytochrome P450 oxidoreductase
POR	POR gene
P450	Cytochrome P450
cyt *c*	Cytochrome *c*
cyt b_5	Cytochrome b_5
NOS	Nitric oxide synthase
FNR	Ferredoxin-$NADP^+$ reductase
Fld	Flavodoxin
FMN domain	FMN-containing flavodoxin-like domain
FAD domain	FAD-containing FNR-like domain plus the connecting domain
P450BM3	*Bacillus megaterium* flavocytochrome P450BM3
MS	Methionine synthase
MSR	Methionine synthase reductase
ER	Endoplasmic reticulum
HO	Heme oxygenase

L. Waskell (✉)
University of Michigan Medical School and VA Medical Center, Ann Arbor, MI, USA
e-mail: waskell@med.umich.edu

J.-J. P. Kim
Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI, USA
e-mail: jjkim@mcw.edu

2.1 Introduction

Cytochrome P450 (P450) electron transport is mediated by a multicomponent monooxygenase system, in which reducing equivalents from NADPH (Nicotinamide Adenine Dinucleotide Phosphate) are transferred to molecular oxygen via one of many cytochrome P450 isozymes [1, 2]. Depending on the cellular location and their redox partners, P450s are generally divided into two major classes, class I and class II. Class l includes mitochondrial and bacterial P450s that use two separate redox partners consisting of an iron–sulfur protein (ferredoxin/adrenodoxin) and a flavin-containing reductase (ferredoxin/adrenodoxin reductase). The class II P450s are microsomal monooxygenases that receive electrons from NADPH-cytochrome P450 oxidoreductase (POR), the founding member of the diflavin reductase family. Both the reductase and the monooxygenases are integral membrane proteins. In addition, there are many minor classes of P450s reviewed in Hannemann, et al.[3], including P450 proteins that are fused to their own diflavin reductase partner in one polypeptide chain, e.g., P450BM3 from *Bacillus megaterium* (see Fig. 2.1). Most mammalian P450s are located in the endoplasmic reticulum (ER). In humans, 50 of 57 P450s are microsomal and the remaining seven are located in mitochondria. The microsomal P450s use a single POR for electron delivery from NADPH. In addition, some microsomal P450s also use cytochrome b_5 (cyt b_5).

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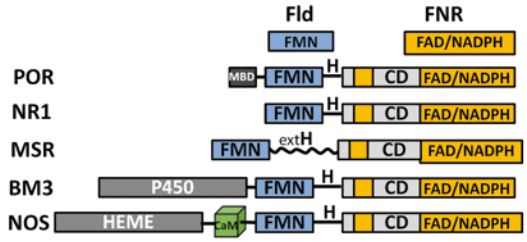

Fig. 2.1 Domain organization of NADPH-cytochrome P450 oxidoreductase (*POR*) and other members of the diflavin oxidoreductase family. *Fld* flavodoxin, *FNR* ferredoxin-NADP$^+$ oxidoreductase, *MBD* transmembrane domain, *H* hinge, *CD* connecting domain, *NR1* novel reductase 1, *MSR* methionine synthase reductase, which contains an ~80 residue extended hinge region (ext**H**) between the FMN domain and CD, *BM3 Bacillus megaterium* flavocytochrome P450, *NOS* nitric oxide synthase, which has a calmodulin-binding region (CaM). Note that the CD consists of two noncontiguous parts of the linear sequence interspersed with the FNR-like domain

POR is a membrane-bound ~78-kDa protein. POR is the prototypic member of the diflavin oxidoreductase family of enzymes that contain one molecule each of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) in a single polypeptide. These enzymes perform a step-down function, i.e., transferring electrons from the two-electron donor NADPH to one-electron acceptors (e.g., heme), with the FAD functioning as a dehydrogenase flavin and FMN as an electron carrier. In other words, NADPH transfers a hydride ion to the FAD, which transfers these two electrons one at a time to the FMN. It is the FMN hydroquinone that is the ultimate electron donor, again one by one, to P450 and other electron transfer partners. Other prominent members of this family are the reductase domains of the nitric oxide synthase (NOS) isozymes (reviewed in [4–7] and flavocytochrome P450BM3 (P450BM3) from *Bacillus megaterium* [8], and the flavoprotein subunits of bacterial sulfite reductase[9], all of which transfer electrons to heme, as well as methionine synthase reductase (MSR), which reduces Cob(II)alamin of methionine synthase [10–12], human cancer-related novel reductase 1 (NR1) [13], pyruvate: NADP$^+$ oxidoreductase from *Euglena gracilis* [14, 15], and reductase Tah18 protein from yeast [16]. The domain structures of these proteins are all similar to that of POR, containing the flavodoxin (Fld)-like and ferredoxin-NADP$^+$ reductase (FNR)-like folds, having similar functions and mechanisms of action (Figs. 2.1 and 2.2).

POR functions to transfer electrons from NADPH to a number of microsomal electron acceptors, including not only P450s but also heme oxygenase (HO) [17], cyt b_5 [18], squalene monooxygenase [19], and possibly indole dioxygenase [20]. In addition, a number of nonphysiological electron acceptors, including cytochrome *c* (cyt *c*), ferricyanide, menadione, and dichloroindophenol, have been used for biochemical characterization of the enzyme. On the other hand, other members of the diflavin oxidoreductase family, including MSR, NOS, and P450BM3, transfer electrons to a single physiological acceptor. For NOS and P450BM3, both the donor and acceptor are located on the same polypeptide (Fig. 2.1). However, both NOS and P450BM3 are dimeric molecules and the reductase domain of monomer 1 reduces the heme domain of monomer 2 and vice versa. The electron acceptors for POR, including the multiplicity of P450s, as well as other protein acceptors listed above, are located in the ER, and the levels of POR are substantially lower than those of its acceptors, with the ratio of POR to P450 in liver ER estimated at 1:5~20 [21–23]. Although the large and diverse family of P450s exhibits a common fold in the vicinity of the heme ligand, each P450 also possesses unique structural features, substrate specificity, and rate-limiting catalytic steps [24, 25]. Thus, electron transfer to all these proteins must proceed in a finely controlled fashion. The question arises as to how POR recognizes and mediates electron transfer to this multiplicity of electron acceptors.

This chapter discusses the mechanism of interaction between P450s and their redox partners, primarily the diflavin oxidoreductase, POR, and cyt b_5. The domain organization and the high degree of conformational changes in POR necessary for the precise orchestration of electron transfer to its >50 different electron acceptors will be highlighted. The complex and controversial role of cyt b_5 as a redox partner for P450 will also be discussed. Details of the reaction of a Class I P450 with an iron sulfur protein are provided in the chapter by Poulos and Johnson.

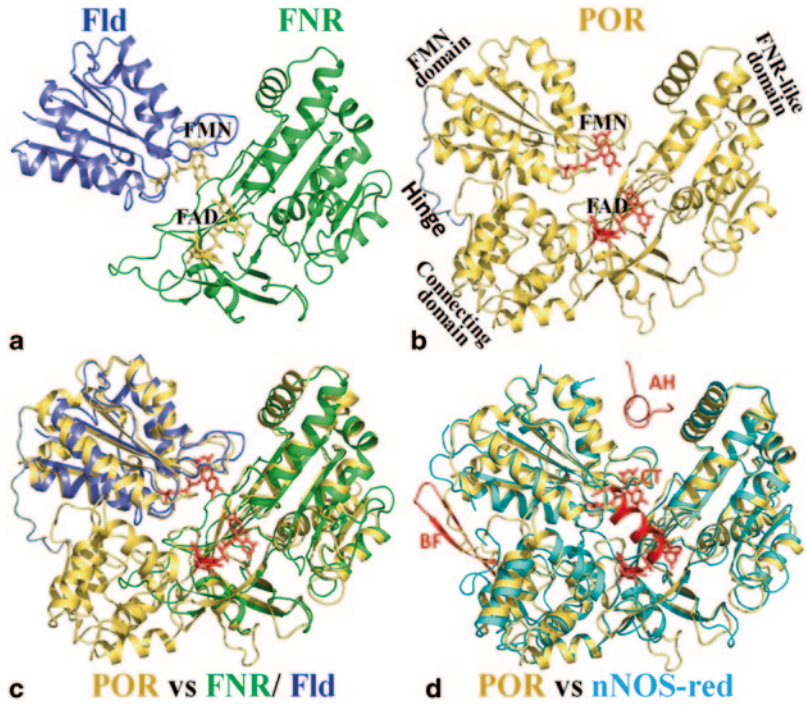

Fig. 2.2 Evolutionary origins of the structures of NADPH-cytochrome P450 oxidoreductase (*POR*) and the neuronal NOS (*nNOS*) reductase domain (*cyan*), shown by overlays of the ribbon structures of *Desulfovibrio vulgaris* flavodoxin (*Fld*) and spinach ferredoxin-NADP-oxidoreductase (*FNR*). **a** Structures of Fld and FNR. **b** POR with flavin mononucleotide (*FMN*) and flavin adenine dinucleotide (*FAD*) highlighted with *red* sticks. The FMN domain, FNR-like domain, connecting domain, and the flexible hinge are marked. **c** Overlay of the structures of Fld, FNR, and POR. The connecting domain and hinge are unique to POR. **d** Overlay of POR and nNOS-red. The nNOS reductase domain [40] contains various regulatory elements, including the autoregulatory insert (*AR*), β-finger (*BF*), and the C-terminal extension (*CT*). They are shown in *red*

2.2 NADPH-Cytochrome P450 Oxidoreductase

2.2.1 Properties of POR Flavins

The ability of flavins to engage in both 1-electron and 2-electron redox chemistry is key to their functions in electron transfer. In POR, they are an essential intermediate between NADPH, a two-electron donor, and the heme of P450, a one-electron acceptor. Furthermore, utilization of two flavins, located in separate domains, provides a mechanism for control of the kinetics of electron transfer by regulating the distance between, and the relative orientation of, the two flavins. The flavin cofactors can exist as the oxidized (ox), one-electron reduced semiquinone (sq), and two-electron, fully reduced (red) forms (Fig. 2.3). Both the semiquinone and the fully reduced forms can exist free in solution as either neutral or anionic forms with pK_a values of 8.5 and 6.5, respectively. Both semiquinones of POR are found as the blue, neutral form in the pH range 6.5–8.5. In this review, the fully reduced forms are referred to as $FMNH_2$ and $FADH_2$. However, the protonation states of the fully reduced forms in POR are unknown. Those of the homologous proteins, FNR and flavodoxin, are anionic and it should be kept in mind that the fully reduced flavins in POR may also be in the anionic forms, $FADH^-$ and $FMNH^-$.

The oxidation and protonation states of the flavins can be distinguished by their distinct visible absorption spectra, which have been invaluable in characterizing the oxidation states of flavoproteins during catalysis [7, 26, 27]. Oxi-

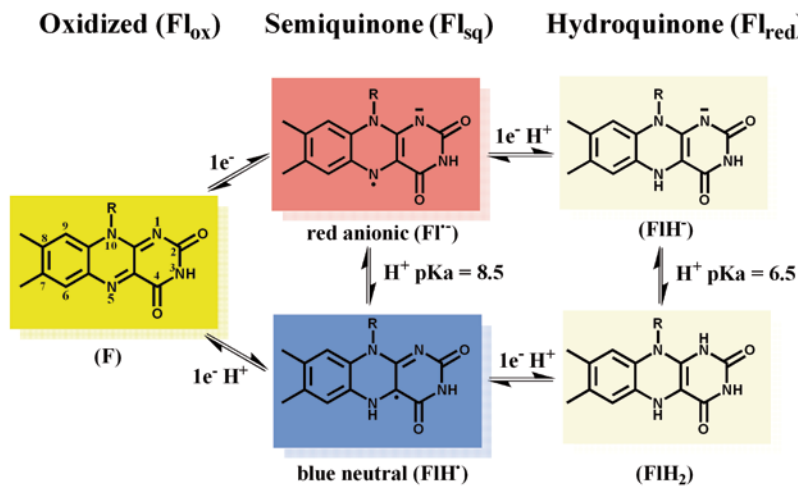

Fig. 2.3 Various redox states of the isoalloxazine ring of flavin mononucleotide (*FMN*) and flavin adenine dinucleotide (*FAD*). The background color for each redox state represents its visible spectrum

dized flavins have broad absorption maxima at approximately 450 and 380 nm. The neutral blue semiquinones are characterized by a broad absorbance between 500 and 700 nm, with maxima in the region between 585 and 600 nm. In POR, the FMN, but not the FAD, semiquinone has a shoulder at 630 nm, which enables discrimination of the FADH• and FMNH• semiquinones and analysis of one-electron transfer reactions between FAD and FMN [27]. The FMNH• semiquinone is air stable, while the FADH• semiquinone is unstable and rapidly oxidizes in air. The stability of the neutral FMNH• semiquinone is likely due to a hydrogen bond between N5 of the FMN and the main chain carbonyl group of a highly conserved glycine residue in a nearby loop (Gly141 in rat POR).

The reduction potentials of the POR flavins have been determined for the rabbit [28], rat [29], and human [30, 31] enzymes. For FMN, $\Delta Eox/sq = -110 \sim -66$ mV and $\Delta Esq/red = -246 \sim -290$ mV; for FAD, $\Delta Eox/sq = -290 \sim -328$ mV and $\Delta Esq/red = -372 \sim -382$ mV. Although there are some variations in reduction potentials between species, the FAD semiquinone/reduced couple always exhibits a low reduction potential (~−380 mV), at or near that of NADPH (−320 mV). Thus, FAD is the low-potential flavin and electron transfer proceeds from NADPH to FAD to FMN to P450 [32]. It should be noted that these reduction potentials have been determined for the solubilized protein in aqueous solution and, that membrane lipids and their compositions may influence the flavin reduction potentials [29].

2.2.2 Redox Cycling of POR Flavins

Figure 2.4 illustrates the overall reaction mechanism by which two-electrons from NADPH are transferred to the one-electron acceptor, ferric P450. Two electrons from NADPH must enter the enzyme as a hydride ion to the FAD, followed by intramolecular electron transfer to FMN. The FMN semiquinone is extremely stable, indicating that it is the hydroquinone FMN that transfers electrons to electron acceptors and that the fully oxidized enzyme form does not accumulate. The POR flavins cycle in a 1-3-2-1 electron cycle (upper half circle in Fig. 2.4a). The air-stable form, FMN•/FAD can be formed from the fully oxidized form during the priming reaction (Fig. 2.4b). At high concentrations of NADPH, the intermediate FMNH2/FAD is reduced to a four-electron reduced form [33, 34]. Since the air-stable semiquinone form is found predominantly in liver microsomes [26], the 1-3-2-1 cycle is likely the major mechanism in vivo. Although the low reduction potential of FAD, near

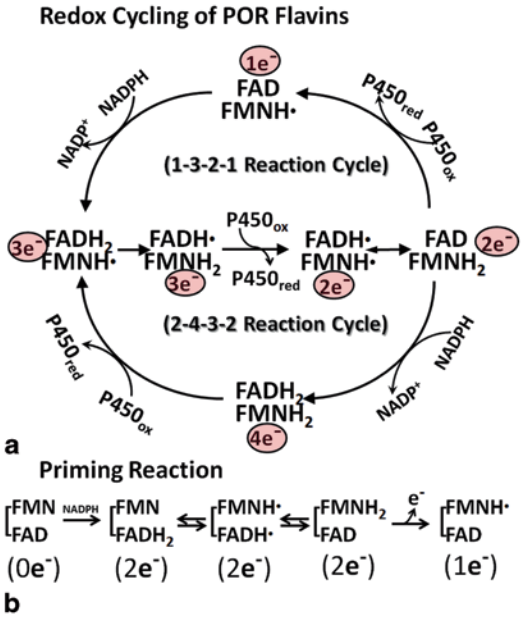

Fig. 2.4 **a** Catalytic cycling of NADPH-cytochrome P450 oxidoreductase (*POR*) flavins. Redox cycling and electron transfer via 1-3-2-1 (*upper half circle*) and 2-4-3-2 (*lower half circle*) electron reaction cycles are shown. The *middle line* is common to both cycles. The air-stable 1e- reduced form (*FAD/FMNH•*) is obtained through the priming reaction. **b** Scheme for the priming reaction, generating the air-stable 1e- reduced form (*FAD/FMNH•*) by reduction of fully oxidized enzyme

or below that of NADPH (−320 mV), suggests that formation of the fully reduced (four-electron reduced) form of the enzyme is thermodynamically unfavorable, the 2-4-3-2 cycle is also possible depending on the NADPH/$NADP^+$ ratio [27].

2.2.3 Domain Structure and Function

As predicted, based on DNA sequence homology [35], POR likely arose from the fusion of two ancestral genes related to the flavodoxin (Fld) and ferredoxin $NADP^+$ reductase (FNR) proteins. This hypothesis has subsequently been confirmed both by site-directed mutagenesis studies and X-ray crystallography [36], confirming the structural and catalytic functions of conserved residues. The domain organization of POR is apparent from the crystal structure of POR, exhibiting domains structurally related to flavodoxin and FNR (Fig. 2.2). Conservation of cofactor binding and catalytic residues is also observed. Furthermore, the fact that boundaries of the domains correspond to exon junctions in the gene encoding the enzyme is additional evidence that POR has arisen from a gene fusion event. The three-dimensional protein structures of spinach FNR, Fld from *Desulfovibrio vulgaris,* and rat POR also strongly support a common ancestor based on the very high structural similarity between the individual domains despite their very different origin [36, 37] (Fig. 2.2). The ability to express the different domains of POR as individual, functionally active proteins, and to successfully reconstitute these domains in vitro to form a functional protein complex of NADPH-cytochrome P450 oxidoreductase activity is additional evidence that POR has evolved as a result of gene fusion event [38, 39].

POR is anchored in the microsomal membrane by a ~56-amino acid N-terminal membrane binding domain (MBD), with the catalytic functions of POR residing in the soluble portion, residues 66–678 (residue numbering is based on rat POR, unless otherwise noted). As shown in Fig. 2.2, the structure of the soluble portion of POR is composed of an FMN-binding domain, which is structurally similar to Fld, and an FAD-binding domain. The FAD domain consists of an FNR-like domain with binding sites for FAD and NADPH and a connecting domain (CD), which is unique to POR and to all members of the diflavin reductase family, including nitric oxide synthases [40]. The CD is composed mainly of α helices that connect (join) the FMN and FNR-like domains. The FMN and FAD domains are linked by a flexible hinge/linker (residues 232–243), consisting mostly of hydrophilic residues.

The presence of a connecting domain and hinge is unique to all members of the diflavin oxidoreductases (Fig. 2.1). Although the amino acid sequences of the connecting domains (CDs) exhibit low (<30%) sequence homology, there is significant structural similarity among connecting domains of different members of the diflavin family (see comparison of POR and nNOS in Fig. 2.2). Both the length and sequence of the hinge are unique for each member of this family.

The hinge plays a crucial role in POR's interaction with its electron transfer partners. It is believed that the hinge and connecting domain are largely responsible for the domain movements that control cofactor binding, interflavin electron transfer, and recognition and electron transfer to the partners (see below).

2.2.3.1 Membrane Binding Domain

POR is anchored to the lipid bilayer of the ER and nuclear membrane by an approximately 60 amino acid MBD. The MBD contains a 23-amino acid stretch of hydrophobic amino acids that presumably spans the lipid bilayer, followed by a stop-transfer sequence, ^{45}RKKKEE^{50}, and a flexible segment susceptible to proteolytic cleavage [41, 42]. Cleavage by trypsin at the Lys56-Ile57 bond releases the POR from the microsomal membrane. The trypsin-cleaved protein is no longer able to transfer electrons to P450, but retains activity towards other electron acceptors such as cyt *c*. Similarly, cyt b_5 is attached to the membrane via a C-terminal MBD that is necessary for electron transfer to P450. Both passive and active roles in P450-mediated catalysis have been proposed for the MBD. Since fusion proteins, such as P450BM3 and the NOS isozymes, do not require the MBD for catalytic activity, the MBD likely serves to localize and possibly restrict movement of POR in the membrane rather than to provide a specific binding site [43–45]. In this case, the precise sequence of the membrane domain would be less important than its ability to insert into the membrane. Substitution of the POR MBD with that of cyt b_5, which has only about 20% sequence identity, but a similar hydrophobicity profile [46], produced a chimeric POR that was able to support CYP17A-mediated P450 activity, but not CYP3A4-mediated testosterone 6β-hydroxylation. Taken together with the observation that the MBD of yeast POR is not required for electron transfer to P450 51 [47], it appears that the MBD may contribute to P450 recognition and binding, but is likely that only one of many POR-P450 interactions may vary depending on the specific P450.

Recently, an interesting function for the MBD has been proposed by Das and Sligar [29], showing that the flavin redox potentials are influenced by the composition of the lipid bilayer. The significance of these altered redox potentials relative to catalysis has not been demonstrated. However, lipid composition, including charge, has been reported to influence rates of P450 metabolism in reconstituted systems [48].

2.2.3.2 FMN Domain

The FMN domain, consisting of residues from 67 to 231 of rat POR, is structurally very similar to the bacterial flavodoxins and consists of a five-stranded parallel β-sheet flanked by five α-helices (Fig. 2.2), with the FMN located at the tip of the C-terminal side of the β-sheet. In addition to the binding site for the FMN prosthetic group, this domain contains residues mediating binding of and electron transfer to acceptors such as cyt *c* and P450. FMN is relatively loosely bound (K_d ~10^{-8} M) and can be reversibly removed from the enzyme by high salt treatment [27, 49]. In the absence of FMN, electron transfer to all acceptors, with the exception of ferricyanide, is abolished.

As observed in Fld, the isoalloxazine ring of FMN is sandwiched between two aromatic groups with Tyr178 coplanar with the *si*- face of the flavin, and Tyr140 located on the *re*-face at a ~60° angle to the isoalloxazine ring [36]. Mutation of Tyr178 to Asp decreases FMN binding to undetectable levels, with an approximately 300-fold decrease in FMN binding affinity, and also disrupts FAD binding [50]. A similar decrease in FMN binding affinity is seen when the homologous residue of human POR, Tyr181, is mutated to Asp [51, 52]; however, FAD binding is not disrupted in the case of the human mutation. Restoration of catalytic activity by FMN demonstrates that the inability to incorporate FMN is the likely basis for the NADPH-cytochrome P450 oxidoreductase deficiency (PORD) phenotype associated with this human mutation. The rate of electron transfer to ferricyanide activity is identical to that seen in the wild-type enzyme, indicating that the hydride transfer is not impaired.

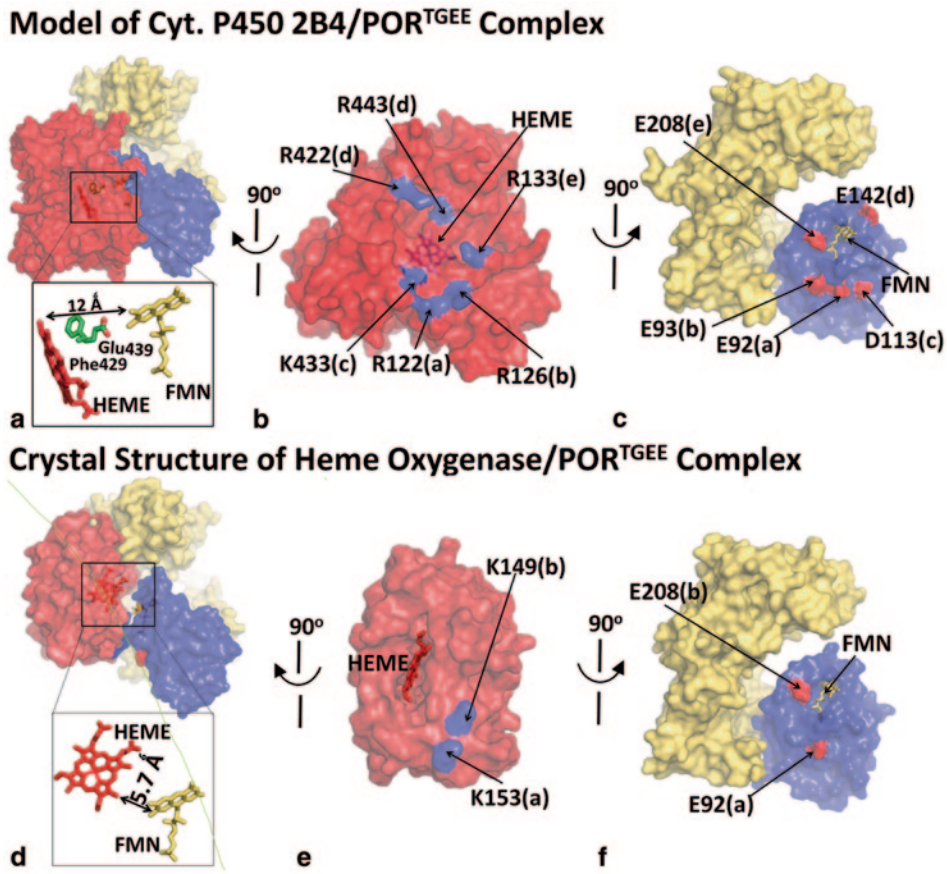

Fig. 2.5 *Top panel*: **a** Model of a complex between P450 and NADPH-cytochrome P450 oxidoreductase (*POR*). A complex of P450 (*red*) and Mol A of the hinge-deletion mutant of POR(ΔTGEE), denoted as POR^{TGEE} [53]); the flavin mononucleotide (*FMN*) domain (*blue*) and flavin adenine dinucleotide (*FAD*) domain (*yellow*)] and an enlarged view showing the relative orientation of the FMN and heme. **b** and **c** Open-book representation of molecular surface at the interface of P450 (**b**) and the FMN domain of POR (**c**). Five salt-bridge pairs are shown with same letters, e.g., Glu142(d) makes salt bridges with both Arg422 (d) and Arg443 (d). *Bottom*: **d** Crystal structure of the complex between POR(ΔTGEE) and heme oxygenase-1 (HO-1). **e** and **f** An open book representation, showing the interface between the two partners. Two salt-bridge pairs are shown. The surface of ΔTGEE that interacts with HO-1 (*Panel F*) is almost the same interface found in the model structure of POR-2B4 (*compare Panels C and F*). The structure of the POR(ΔTGEE)-HO-1 complex supports the validity of the model structure of POR-P450 2B4.

2.2.3.3 Role of the FMN Domain and Connecting Domain in the Cytochrome P450 Interaction

The negatively charged surface of the FMN domain can interact with the basic concave proximal face of P450 in the vicinity of the buried heme ligand [53–56]. This region of P450 contains overlapping binding sites for POR and cyt b_5 [54]. A model of a putative complex of P450 2B4 and POR shows the total contact area between the two molecules to be ~1500 Å^2, of which 870 Å^2 is located between the FMN domain and P450 [53]. A number of charge pairing and van der Waal's interactions have been implicated in binding of P450 to POR, indicating that both electrostatic and hydrophobic interactions are necessary for the complex formation (Fig. 2.5).

The FMN domain has conserved patches of acidic residues involved in the electrostatic interactions with its electron transfer partners, and these interactions are specific for each electron transfer partner. Cross-linking experiments

suggest that acidic residues in the FMN domain (^{207}Asp-Asp-Asp^{209} and ^{213}Glu-Glu-Asp^{215}) contribute to binding of cyt *c*; however, cross-linking of these residues to P450 could not be demonstrated [57, 58]. Mutagenesis studies have demonstrated the importance of Glu213 and Glu214 in electrostatic interactions with oxidized and reduced cyt *c*. The ^{213}Glu-Glu-Asp^{215} cluster does not affect P450 binding or activity, highlighting the distinct binding modes for these two partners [59]. Chemical modification and antibody labeling experiments have also suggested that the loop containing residues 110–119 in POR, located on the opposite face of the protein, can also contribute to P450 binding and catalysis (reviewed in [60]). Site-directed mutagenesis of Asp113, Glu115, and Glu116 improves catalytic efficiency of cyt *c* reduction, but destabilizes the POR-CYP2B1 complex [61]. A variety of chemical modification and mutagenesis studies, reviewed by Hlavica et al. [62] and Im and Waskell [55], have provided evidence implicating basic residues in the C-helix of P450 in electrostatic interactions with POR and cyt b_5. Site-directed mutagenesis studies have identified seven basic and hydrophobic amino acids (Arg122, Arg126, Arg133, Phe135, Met137, Lys139, and Lys433), all except Lys433 located in the mobile C-helix and C–D loop, as important for both cyt b_5 and POR binding [54]. Mutations to proline of residues in the linker between the two flavin domains also increased the cyt *c* reduction activity, presumably by favoring the open conformation of POR [63]. The hydrophobic amino acid residues Val267 and Leu270 on the proximal site of CYP2B4 also contribute to POR recognition, perhaps indirectly through a conformational change [64]. Although the electron transfer is presumed to occur within a 1:1 POR:P450 complex [65], the presence of higher-order complexes contributing to catalysis has been suggested [23, 66, 67]. The contribution of these higher-order complexes to catalysis in microsomes is not clear. However, it is likely that multiple P450s may associate to POR during the selection process in the course of catalysis as an encounter complex (see Sect. 2.3.2).

2.2.3.4 The FAD Domain

The FAD domain of POR is composed of the connecting domain (CD) and the FNR-like subdomain, which binds FAD and NADPH (Figs. 2.1 and 2.2). The FNR-like subdomain sequence consists of residues 267–325 and 450–678, interspersed with the CD (residues 244–266 and 326–450). Conserved residues necessary for FAD and NADPH binding, as well as for hydride transfer, are localized in this FNR-like subdomain. Unlike FMN, FAD is tightly bound to the reductase with a K_d less than 1 nM. Removal of FAD requires treatment with a high concentration of chaotropic agent that leads to substantial polypeptide unfolding, providing further evidence for the independence of the two domains [68–70]. Residues comprising the FAD binding site include ^{455}YYSIASS^{461}, ^{471}ICAVAVEY^{478}, and ^{488}GVAT^{491}. Although Trp677 is stacked against the *re*-face of the FAD, removal of this residue does not have a significant effect on FAD content; the role of this residue in catalysis is discussed below. Major determinants of FAD binding are Arg454, which stabilizes the negative charge of the FAD pyrophosphate, and Tyr456, which is positioned at a 60° angle to the *si*-face of the isoalloxazine ring and whose phenolic hydroxyl group forms a hydrogen bond with the ribityl 4′-hydroxyl [36, 71]. An unexpected finding for residues that influence FAD-binding was revealed in a human pathogenic mutant, Val-492Glu (rat enzyme numbering, V489), which has less than 1 % of wild-type FAD content (see Sect. 2.6).

2.2.4 Mechanism of Catalytic Action

2.2.4.1 Hydride Transfer

POR transfers the *pro-R* hydrogen from NADPH to FAD as a hydride ion. Residues essential for this hydride transfer include Ser457, Asp675, and Cys630, all of which are located in close proximity to the redox-active N5 of FAD and form a hydrogen bonding network that is disrupted upon

binding of the nicotinamide moiety of NADP(H) [72–74]. Replacement of these side chains with aliphatic groups decreases catalytic activities by up to three orders of magnitude. Ser457 and Asp675 interact with the nicotinamide group of NADP(H) and orient the C4 atom of the nicotinamide ring in a position for optimum hydride transfer. Cys630 is also within van der Waals distance from the nicotinamide C4 and can stabilize the carbocation formed during hydride transfer [74]. In addition, the hydroxyl side chain of Ser457 is located ~4 Å away from the flavin N5 and on the same plane as the flavin ring, in a position to stabilize the semiquinone form of FAD, and replacement of Ser457 with alanine decreases the FAD/FADH• redox potential [72].

The penultimate Trp677 residue plays a pivotal role in catalysis by controlling NADP(H) binding and release [74]. In the structure of the wild-type reductase in complex with $NADP^+$, the indole ring of Trp677 is situated at the *re*-face of the FAD, where the nicotinamide ring of NADPH would bind to transfer its *pro*-R-hydrogen as a hydride ion. Furthermore, in the structure of the wild-type enzyme, the binding site for the AMP-pyrophosphate half of the $NADP^+$ is clearly shown, while the ribose-nicotinamide moiety is disordered. However, crystal structures of a POR mutant lacking the indole ring by deletion of the two last C-terminal residues (Trp677 and Ser678), or mutation of Trp677 to glycine (Trp677Gly), reveal that the nicotinamide ring is situated at the *re*-face of the FAD, replacing the indole ring of Trp677, with a tilt of ~30° between the planes of the two rings, poised to transfer the hydride ion [74]. Thus, in the wild-type protein, the indole ring of Trp677 presumably moves away from the isoalloxazine ring of FAD, allowing the nicotinamide ring to interact with the flavin for hydride transfer to occur. In pea FNR, the homologous residue, Tyr308, is also displaced by the nicotinamide ring [75, 76].

Mutagenesis and crystallographic studies have revealed the bipartite nature of NADP(H) binding and provide an explanation of the marked preference of POR and FNR for the cofactor NADPH. The primary determinant for discrimination between NADH and NADPH is the 2′-phosphate group present on NADPH, but not NADH. Kinetic studies show that this 2′-phosphate of NADPH, binding as the dianion, contributes 5 kcal of binding energy through interactions with enzyme groups, with a major contribution with Arg597 accounting for ~3 kcal of binding energy. Lys602 and Ser596 also contribute to binding [77]. This tight binding of the 2′-phosphate is essential to compensate for the repulsive interactions between the nicotinamide and the indole ring of Trp677. When Trp677 is present, binding of the 2′-phosphate stabilizes cofactor binding sufficiently to allow the nicotinamide to displace Trp677. In the absence of Trp677, the nicotinamide can bind readily without any contribution from the 2′-phosphate and the enzyme is able to utilize NADH as the hydride donor. Furthermore, in the absence of Trp677, the enzyme is unable to displace oxidized nicotinamide after hydride transfer and catalytic efficiency with either NADH or NADPH is decreased due to rate-limiting product release [74, 78, 79], indicating that movement of Trp677 is required for both cofactor binding and release.

These studies indicate a requirement for structural changes, in addition to Trp677 movement, for regulation of NADP(H) binding and release. While movement of Trp677 back into the nicotinamide binding site (*re*-face of the FAD isoalloxazine ring) displaces the nicotinamide ring, additional movements are necessary to disrupt the strong binding of the 2′-phosphate. Local movements of the ^{631}GDAR^{634} loop (Asp632 loop), located near the FAD, may be coupled with Trp677 movement to allow NADPH binding and $NADP^+$ release [80]. Comparison of the structure of the $NADP^+$ -bound wild-type enzyme with that of a mutant POR with an engineered disulfide bond between the two flavin domains and lacking bound $NADP^+$, shows a movement of this Asp632 loop. Thus, Xia et al. have proposed that Asp632 loop movement, in concert with Trp677, controls at least in part NADPH binding and $NADP^+$ release [80], and the details are discussed below in Sect. 2.5.

2.2.4.2 Interflavin Electron Transfer

POR intramolecular electron transfer occurs directly from FAD to FMN. In rat and human POR [36, 81], the distance between the

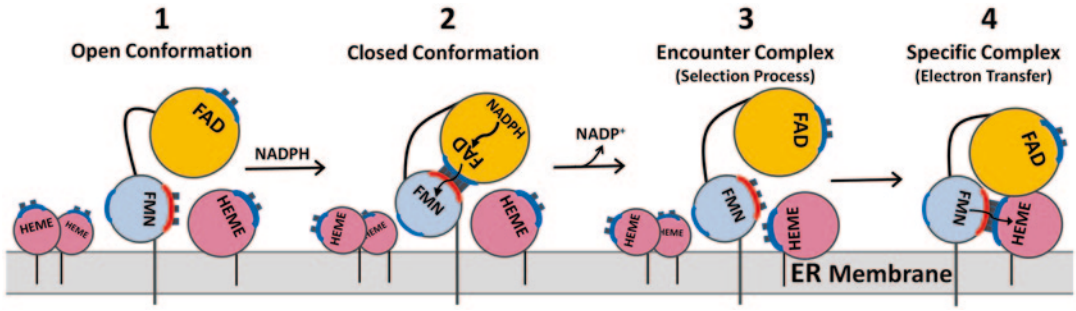

Fig. 2.6 A cartoon representation of a model for POR-P450 complex formation in the endoplasmic reticulum (*ER*) membrane. Flavin mononucleotide (*FMN*) domain, flavin adenine dinucleotide (*FAD*) domain, and P450s are shown in blue, yellow, and red balls, respectively. (1) Multiple P450s exist in the ER membrane. Nucleotide binding favors formation of the closed form, similar to the one found in the crystal structure [36]. (2) Upon binding to pyridine nucleotide (*NADPH*), the enzyme adopts the closed form. In the closed form, hydride transfer, interflavin electron transfer, and release of NADP+ occur, resulting in formation of the open form of the enzyme. (A scheme for detailed conformational changes occurring during this process is shown in Fig. 2.7.) (3) The open form of POR associates with P450 in an encounter complex. (4) Further conformational adjustments occur to align the flavin and heme groups in an optimal conformation for electron transfer, and the cycle repeats. (Figure adopted and modified from [86])

dimethylbenzene edge of the isoalloxazine rings of FAD and FMN is ~4 Å, and the planes of the FAD and FMN rings are inclined relative to each other at an angle of ~150°, an orientation that favors orbital overlap between the extended π–π systems of the flavin isoalloxazine rings [74]. This arrangement of the two flavins is expected to result in very fast and efficient interflavin electron transfer, up to 10^{10} s^{-1} using Dutton's ruler [82]. However, the experimentally observed electron transfer rate has been measured to be only ~50 s^{-1} [83, 84], suggesting that electron transfer is gated by some other process. The nature of the conformational movements controlling the rates of interflavin as well as flavin to heme electron transfer is discussed below.

2.2.4.3 Electron Transfer from FMN to Heme

The FMN domain functions both to accept electrons from the reduced FAD and to transfer those electrons to P450. Thus, precise and specific interactions between the FMN and FAD domains within POR, and between the FMN domain and P450 are required. This means that the FMN domain must be able to recognize both the FAD domain and P450. Separation of the two flavin domains is essential for this sequential electron transfer process. The FMN domain has a strong molecular dipole formed by anionic residues surrounding the flavin isoalloxazine ring [85]. This convex anionic surface is involved in the specific docking with the heme protein. Little is known about the mechanism through which POR selects one of many electron transfer partners and it is likely that multiple protein conformations and binding sites are probed in the selection process. Figure 2.6 presents a scheme incorporating current hypotheses regarding formation of a productive POR-P450 electron transfer complex. Beginning from a pool of P450s in the ER membrane, in which multiple P450s exist, a selection process must occur by which one P450 binds in a more favorable conformation. A proposed sequence of events is as follows: (1) NADPH binds to the open form of POR, resulting in a closed conformation of POR. (2) In this closed conformation, hydride transfer and interflavin electron transfer occur, followed by $NADP^+$ release, resulting in an open conformation of POR. (3) This open form of POR is now capable of forming an eventual productive complex. It should also be noted that POR will favor substrate-bound ferric P450s compared to substrate-free P450s. Substrate binding increases the redox potential of the P450, makes the electron transfer reaction thermodynamically feasible, and prevents inappropriate reduc-

tion of P450. Substrate binding may also induce conformational changes on the proximal surface that favors POR binding. (4) A loosely bound encounter complex is formed [87]. (5) Further conformational changes at the interface are necessary to produce the electron transfer complex, in which the flavin and heme are appropriately positioned for electron transfer [87]. For a more detailed discussion on general protein–protein interactions, see the latter part of this chapter. The requirements for cyt *c* binding are most likely less stringent than those for P450, and kinetic studies suggest the presence of more than one binding site for cyt *c* [88]. In contrast, the mechanism of electron transfer to small molecule acceptors such as dichloroindophenol or ferricyanide presumably involves random collisions followed by electron transfer.

A model for a docked POR-P450 complex (POR-P450 2B4) based on mutagenesis data with the open conformation of the POR hinge mutant (four amino acid deletion in the hinge between two flavin domains) by Hamdane et al. [53] indicates that the FMN domain interacts with the concave basic proximal face of P450. The planes of the heme and FMN are almost perpendicular to each other, and the shortest distance between the heme and flavin cofactors is about 12 Å (Fig. 2.5). However, two residues of P450 2B4, Phe429, and Glu439, lie in between the two cofactors, suggesting that these might serve to facilitate electron transfer between the FMN and heme. In the structure of the complex between the heme and FMN-binding domains of bacterial cytochrome P450BM3, the relative orientation of the two cofactors is similar to that found in the model structure, but the distance between the FMN and heme is slightly longer (~18 Å) [89], indicating the validity of the model structure. Recently, the crystal structure of the complex between the four-residue hinge deletion mutant of POR (ΔTGEE) and rat heme oxygenase 1 (HO-1) has been determined [90]. The complex structure reveals that the distance between FMN and the heme is ~6 Å. However, the surface of ΔTGEE that interact with HO-1 is almost identical to that found in the model structure of POR-2B4, although the interface area is smaller, since HO-1 is a smaller molecule than P450 (Fig. 2.5). This finding is consistent with the argument that the model structure of the POR-P450 2B4 complex is an appropriate initial model for further experimental design.

2.2.5 Domain Movement and Electron Transfer in POR

As stated above, the relatively slow rate of interflavin electron transfer suggests a gating mechanism. Crystal structures of various POR proteins, including the rat [36], human [81], and yeast PORs [91], and their various mutant proteins [74], clearly demonstrate that the enzyme molecule consists of two flavin-binding domains, and that the two cofactors are juxtaposed to each other with their dimethyl benzene rings facing one another, with the closest distance being ~4 Å. Although this arrangement of the two flavin domains ("closed" conformation) is optimal for electron transfer between the two flavins, i.e., from FAD to FMN, it is incompatible with interaction of the FMN domain with P450, the physiological electron acceptor. In the closed conformation, the acidic residues located in the FMN domain and shown to affect electron transfer to P450 by mutagenesis studies [59] are not exposed to solvent, and therefore cannot interact with P450. In addition, the crystal structure of a complex between the heme and FMN-binding domains of P450BM3 provides structural insight into how these two domains interact with each other [89]. In this structure, the FMN dimethylbenzene ring is oriented toward the proximal face of the heme of P450 BM3, suggesting that POR must interact with P450 in a different conformation than the closed conformation observed in the wild-type POR crystal structure.

There are several lines of evidence from crystallographic studies, demonstrating that the two flavin domains are mobile. Superposition of the structures of wild-type and various point mutant structures of rat POR has shown that the relative orientation of, and distance between, the two flavin domains are variable, with the closest flavin–flavin distance ranging from 3.9 to

5.8 Å, suggesting small, but significant domain movements in solution [74]. Moreover, in the crystal structure of the flavoprotein subunit of *E. coli* sulfite reductase, electron density for the entire FMN domain is completely disordered, again suggesting movement of the FMN domain relative to the rest of the polypeptide [92]. The most direct demonstration of a large-scale domain movement and a transition from a closed to an open conformation comes from the crystal structures of mutant POR proteins. A POR variant with a four amino acid deletion in the hinge region that links the two flavin domains has been crystallized in three different extended conformations (open state), in which the distance between FAD and FMN cofactors ranges from 30 to 60 Å [53]. The mutant is defective in its ability to transfer electrons from FAD to FMN. However, when FMN is reduced chemically, the mutant POR is capable of reducing P450 2B4. The authors infer that a similar domain movement controlled by the hinge occurs in the wild-type enzyme during its catalytic cycle, enabling the FMN domain to adopt an open conformation capable of interacting with its physiological partner, cytochrome P450. Aigrain et al. have also seen an open conformation in the crystal structure of a yeast-human chimeric POR [93]. A different, but complementary approach has been used by Xia et al. [80], in which an engineered disulfide linkage between the two flavin domains locks POR in a closed conformation unable to interact with P450. Indeed, the mutant exhibits substantially decreased inter flavin electron transfer and is essentially unable to catalyze the P450-dependent monooxygenase activity. Reduction of the disulfide linkage restores the ability of the mutant to support both interflavin electron transfer and reduction of its redox partners, consistent with domain movements being required for the FMN domain of POR to interact with both the FAD domain and P450, i.e., shuttling between the two redox-active partners.

In addition, several solution studies provide evidence for large domain movements of POR in catalysis. Hay et al., demonstrate, using electron-electron double resonance methods, that POR exists in multiple conformations in a continuum of a conformational landscape that is changed by nucleotide binding [94]. Using a combination of nuclear magnetic resonance (NMR) and small-angle X-ray scattering (SAXS) methods, Ellis et al. [95] have shown that the oxidized human POR exists in solution as a mixture of approximately equal amounts of two conformations, one consistent with the crystal structure (closed form) and one a more extended structure, which presumably is required for interaction with its electron transfer partners (open form). In addition, the relative contributions of each conformation at equilibrium are affected by the binding of NADP(H), with the nucleotide bound form favoring the closed form. On the other hand, Vincent et al. [96] have recently employed high resolution NMR measurements with residue-specific ^{15}N relaxation and $^{1}H-^{15}N$ residual dipolar coupling data to show that oxidized POR in solution in the absence of bound nucleotide exists in a unique and predominant conformation resembling the closed conformation observed in the crystal structure. However, at present more data are accumulating for the predominance of the closed form when the nucleotide is bound. Pudney et al. [97] have demonstrated, using a combination of fluorescence resonance energy transfer and stopped flow methods, that open and closed states of POR are correlated with key steps in the catalytic cycle, i.e., NADPH binding induces closing of POR and reduction of flavins and/or $NADP^+$ release induces opening of POR. Recently, Huang et al. have shown, using small angle X-ray scattering and small angle neutron scattering together with site-directed mutagenesis, that POR in solution exists in equilibrium between a compact (closed) conformation and an extended (open) conformation and that this equilibrium is linked to nucleotide binding and redox state [63]. Currently, it is generally agreed that the closed conformation is favored when the NADP(H) is bound and the FAD is oxidized; and that the enzyme adopts an open conformation ready to transfer electrons to P450, i.e., when the enzyme is reduced and $NADP^+$ has been released (see Fig. 2.6).

In summary, there is mounting evidence that POR must undergo several different types of

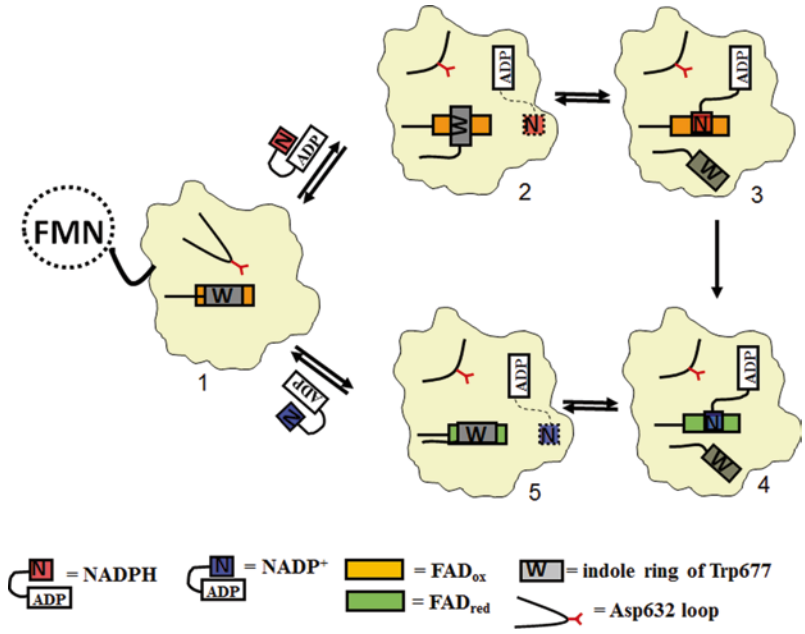

Fig. 2.7 Schematic illustration of conformational changes occurring in the flavin adenine dinucleotide (*FAD*) domain upon NADPH binding to and $NADP^+$ release from NADPH-cytochrome P450 oxidoreductase (*POR*). *Stage 1*: NADPH enters oxidized POR (modeled after the structure of the disulfide cross-linked mutant, which lacks bound NADP(H)) [80]. The open-closed state of POR at this stage is not known, but is most likely in an open form. Stages 2–5 are most likely in the closed form. Therefore, for clarity, the flavin mononucleotide (*FMN*) domain is not shown. *Stage 2*: NADPH initially binds to the enzyme via its AMP-PPi moiety with the interaction between the negative charges of pyrophosphate and 2′-phosphate with the positive charges of several arginine residues in the binding pocket (see text). As the AMP-PPi half of NADPH binds, and the Asp632 loop moves, allowing the ribose–nicotinamide moiety to extend to search for the proper binding site for hydride transfer, while keeping the AMP-PPi anchored. At this stage, the indole ring of Trp677 also rotates to be ready to move away from the flavin ring. *Stage 3*: The indole ring moves away to make room for the nicotinamide ring to bind, as the nicotinamide ring moves in at the *re*-side of the isoalloxazine ring. *Stage 4*: Hydride transfer occurs, FAD is reduced, and NADPH becomes $NADP^+$. It is most likely that interflavin electron transfer occurs at this stage. *Stage 5*: Once the FAD is reduced and nicotinamide is oxidized, the oxidized nicotinamide ring moves out, with the concomitant return of the indole ring to the *re*-face of the FAD ring. At this stage, the Asp632 loop moves back closer to where the AMP-PPi of $NADP^+$ lies, causing steric hindrance as well as electrostatic repulsion, resulting in dissociation of the cofactor from the enzyme. POR now returns to stage 1 and the cycle repeats. (Figure adopted and modified from [80])

conformational changes during catalysis. Hubbard et al. have shown that, upon binding of NADPH, the C-terminus of POR including the aromatic residue Trp677 undergo significant conformational changes [74]. In addition, comparison of the structure of POR with and without bound $NADP^+$ suggests that the movement of the loop containing Asp632 is necessary for binding of the nicotinamide moiety of NADPH to the *re*-face of the FAD isoalloxazine ring. Given these results, suggesting that Trp677 and the Asp632 loop movements occur together, Xia et al. [80] have proposed a scenario for coordinated conformational changes that occur during NADPH binding, hydride transfer and $NADP^+$ release (Fig. 2.7). Since NADPH-binding and Trp677 movement precede hydride transfer, these steps and the subsequent interflavin electron transfer step must occur with the enzyme in the closed conformation. This is followed by a large-scale domain movement to the open conformation that is necessary for interaction with P450. This large movement must be tightly coordinated with electron transfer to prevent reactions with oxygen and production of superoxide. It is most likely that a similar sequence of conformational changes

would also take place in other members of the diflavin oxidoreductase family. However, details of the mechanism by which the large-scale domain movements are coordinated to movements of loops and individual amino acids remain to be established. Furthermore, at this time it is unknown whether stochastic domain movements play a role in the mechanism of action of POR or whether they are strictly controlled.

2.2.6 Human POR Deficiency

Several lines of evidence exist in different biological systems demonstrating the essential cellular functions of POR-dependent P450 activity. The entire POR gene deletion is lethal in yeast and *Caenorhabditis elegans* due to impaired P450-dependent biosynthesis of ergosterol and an as-yet unidentified lipid, respectively [98–100]. Global deletion of murine microsomal POR produces multiple developmental defects and embryonic lethality. Neural tube, cardiac, eye, limb, and vascular defects are seen in homozygous null embryos, as well as a failure of development, which have been ascribed to defects in cholesterol and retinoid metabolism [101, 102].

The ability to delete POR in a tissue-specific manner has provided further insights into the diverse physiological functions of POR, both in metabolism of endogenous substrates and xenobiotic metabolism. Liver-specific ablation of *POR* gave rise to massive lipid accumulation and hepatomegaly, in the presence of decreased serum cholesterol and triglyceride levels, suggestive of defects in regulation of hepatic lipid metabolism. Consistent with the central role of hepatic POR in drug metabolism, liver-specific ablation of POR decreased metabolism and/or clearance of xenobiotics [103–106]. Current developments of various mouse models were presented at a symposium in Experimental Biology 2012 (Symposium Report published [107]).

Since the first report of four individuals with POR deficiency [108], numerous reports worldwide have been published, describing the varying phenotypes associated with this syndrome. A total of over 2000 single nucleotide polymorphisms have been described in the human POR gene (www.ncbi.nlm.nih.gov/snp), encompassing over 150 missense mutations (including premature terminations), over 10 frame shift/deletion/duplication mutations, and 9 splice site variants. Mutations affecting transcription have also been identified and interpreted in terms of the POR promoter structure [109, 110]. Detailed information on POR deficiency with a clinical focus can be found in several excellent reviews [111–113].

Mutations in human POR that significantly disrupt cholesterol biosynthesis and/or steroidogenesis have been shown to result in POR deficiency, characterized by Antley–Bixler syndrome and disordered steroidogenesis [108, 114]. Clinical findings vary greatly in POR deficiency, ranging from severe skeletal malformations associated with the Antley–Bixler syndrome and congenital adrenal hyperplasia to relatively mild hormonal dysregulation. In general, the most severe phenotypes are associated with largest disruptions in ability of POR to support P450-dependent activity [112]. CYP17A1 is known to be particularly sensitive to perturbations in electron transfer, with 17,20 lyase activity favored over 17α hydroxylase activity in the presence of cyt b_5 [116]. Therefore, disordered steroidogenesis is a prominent feature of POR deficiency, which distinguishes it from the Antley–Bixler syndrome with normal steroidogenesis associated with mutations in the fibroblast growth factor receptor 2 (FGFR2) gene. POR deficiency is also associated with congenital adrenal hyperplasia without Antley–Bixler abnormalities. However, recent studies show that conditional deletion of the POR gene in osteoprogenitor cells affects long bone and skull development in mice, recapitulating Antley–Bixler syndrome [117]. These results also suggest an apparent link between the POR and FGFR signaling pathways.

Sequence homology and mapping of missense mutations onto the POR crystal structure have allowed identification of the functions of several missense mutations. Tyr181Asp, Arg457His, Tyr459His and Val492Glu mutations result in low cyt *c* and CYP17A1 activities; Tyr181Asp causes decreased affinity for FMN-binding [51,

118], and Arg457His and Val492Glu cause decreased FAD-binding affinity [108, 119]. The results of Tyr181Asp and Tyr459His mutations are entirely consistent with the hypothesis that the aromatic residues are required for binding of FAD and FMN [81, 119]. Furthermore, the crystal structures of human wild-type and two variants (Val492Glu and Arg457His) have been determined [81]. The overall 3D structures of Arg457His and Val492Glu variants are similar to wild-type; however, there are subtle, but significant differences, including local disruption of hydrogen bonding and salt bridging involving the FAD pyrophosphate moiety, leading to weaker FAD binding, an unstable protein, and loss of catalytic activity, all of which can be rescued by cofactor addition. Thus, riboflavin therapy may prevent or rescue from POR dysfunction patients with these mutations [81, 119].

Although mutations that dramatically decrease POR activity are rare, other polymorphisms, such as Ala503Val, are quite common and there is interest in the effects of these variations on inter-individual variability in drug metabolism [120–122]. The complexity of this effort may be illustrated by studies on the Ala503Val mutant, which has an allele frequency of ~27% [120–122]. In view of the high frequency of this allele, several studies have attempted to assess the contribution of this mutation to inter-individual variation in drug metabolism. Variable results are reported, depending on the P450, the substrate, and the assay systems employed [123–126]. It is increasingly apparent that the effects of POR variants on P450-mediated metabolism require examination of each P450-POR pair and possibly each substrate separately, with further complications introduced by the membrane environment.

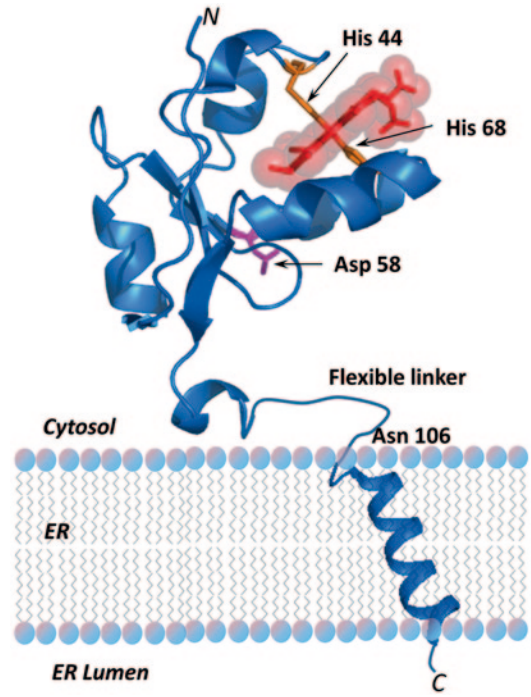

Fig. 2.8 Structure of the heme domain and flexible linker of cyt b_5 and a model of the transmembrane domain in a bilayer

2.3 Interaction Between Cytochrome b_5 and Cytochrome P450

2.3.1 Properties of Cytochrome b_5

Cytochrome b_5 (cyt b_5) is a 134 amino acid membrane-bound electron transfer heme protein that is anchored to the ER membrane by its COOH terminus. The soluble heme domain and membrane anchor are connected by a ~14 amino acid random coil linker [54, 127–129] (Fig. 2.8). It also exists as a soluble protein in red blood cells, where it transfers electrons from cyt b_5 reductase to hemoglobin. Its membrane-bound form provides electrons for the biosynthesis of lipids including plasmalogens, cholesterol, and long-chain fatty acid desaturation [127, 129]. In these reactions, cyt b_5 reductase provides the electrons to cyt b_5. A cyt b_5 domain also exists as a fusion protein in mitochondrial sulfite oxidase, Δ5- and −Δ6 fatty acid desaturases in animals, yeast inositol phosphorylceramide oxidase, plant nitrate reductase, Δ9-fatty acid desaturases in baker's yeast, NADH cyt b_5 oxidoreductase in animals, and flavocytochrome b_2 in yeast mitochondria [127, 130]. A closely related mitochondrial cyt b_5 has also been described. The human mitochondrial cyt b_5 has been shown to provide electrons to an amidoxime-reducing electron transfer chain. It reduces a molybdenum containing enzyme,

which, in turn, directly reduces the N-hydroxylated substrate [131].

The interaction of cyt b_5 and P450 has been well established. However, it remains a complex and controversial topic that has been reviewed previously [54, 127, 132]. In vitro in reconstituted systems, as well as in vivo in the mouse knockout and the mouse with a conditional hepatic deletion of cyt b_5, the effects of cyt b_5 on P450 are contradictory and incompletely understood [132–136]. In purified reconstituted systems, cyt b_5 has been observed to stimulate the activity of some P450s (CYP2B4, CYP2E1, CYP2B1, CYP4A7, CYP2A6, CYP2C19, CPY3A4, CYP17A). In contrast, cyt b_5 has no significant effects on the activity of P4501A2 and 2D6 [137]. Reports have also appeared of inhibition of P450 activity by cyt b_5 [132, 138, 139]. In vivo disposition of drugs in the total body cyt b_5 knockout mouse and in the conditional hepatic cyt b_5 deletion mouse were also complex. The metabolism of some drugs was decreased, while degradation of other drugs was not affected [136, 140]. This chapter will primarily emphasize advances in our understanding of the P450-cyt b_5 interaction that have occurred over the past decade. More than four decades ago, it was shown that cyt b_5 had the ability to decrease the concentration of oxy Fe^{+2} P450 in hepatic microsomes upon addition of NADH to an NADPH-containing reaction mixture, which was consistent with the ability of cyt b_5 to transfer electrons to P450. The molecular basis of this interaction between cyt b_5 and P450 has intrigued investigators ever since [141].

2.3.2 General Characteristics of Interprotein Interactions

Before proceeding with the specifics of the P450-cyt b_5 interaction, the properties of interprotein interactions in general will be presented to provide the framework for the discussion of the P450-cyt b_5 interaction and to help appreciate the P450-POR interaction discussed in the previous section of this chapter. In order for electron transfer to occur between proteins, they must come into contact [142]. Complexes formed between electron transfer proteins typically are weak, on the order of millimolar to micromolar affinities [143]. This weak affinity allows specific but not too perfect binding, so that redox partners can bind, but then readily dissociate and proceed to recycle. If proteins were free in solution, a collision would require a 3D search for the electron transfer site. However, in redox proteins and many other protein complexes, the docking sites have been designed to increase the efficiency of the interaction by employing electrostatic steering and structural complementarity. Electrostatic forces are inversely proportional to the square of the distance between the charged surfaces and are effective over distances up to 25 Å. Structural complementarity is also a major driving force for protein binding. In the case of P450 and its redox partners, cyt b_5 and P450 reductase, the binding of the proteins to the membrane is also hypothesized to decrease the search for the docking site from three to two dimensions. Although electrostatic forces enhance the association rate of proteins, they are considered to result in an "encounter complex," which may not be the optimal electron transfer complex. Following formation of the "encounter complex," short-range diffusion occurs at the interface with sidechains and backbone atoms of residues at the interface undergoing rapid motions to identify a suitable electron transfer complex [87]. Electron transfer occurs rapidly over distances of 14 Å or less, thereby assuring that electron transfer is faster than the usual millisecond bond-breaking at the catalytic site [142]. The 14 Å distance is between the edges of the entities, such as heme and the isoalloxazine ring, exchanging electrons. Quantum chemical calculations suggest that the wave function of a free electron localized at a redox center, for example heme, extends beyond the cofactor in all directions, while decaying exponentially into the electrically insulating amino acid medium [144]. To maintain charge neutrality, proton transfer often occurs essentially simultaneously with electron transfer.

Clackson and Wells have shown that an average of 10–30 residues from each protein are in contact in crystal and NMR structures at an interprotein interface, but that only three to four

amino acid pairs contribute the majority of binding energy to the complex [145]. Site-directed mutagenesis is the major tool employed to investigate which amino acids are most critical. Often, the key residues are found near the center of the interface while the more peripheral residues contribute less binding energy to complex formation, but most likely serve to occlude bulk solvent from the hot spot. Hydrophobic and ionic interactions, as well as hydrogen bonds, are all typically found in a protein interface, although one type of interaction may dominate [146]. It has also been noted that redox proteins that are reactive toward multiple partners, such as cyt b_5 and P450 reductase, employ binding sites that are able to accommodate a variety of molecular surfaces [147].

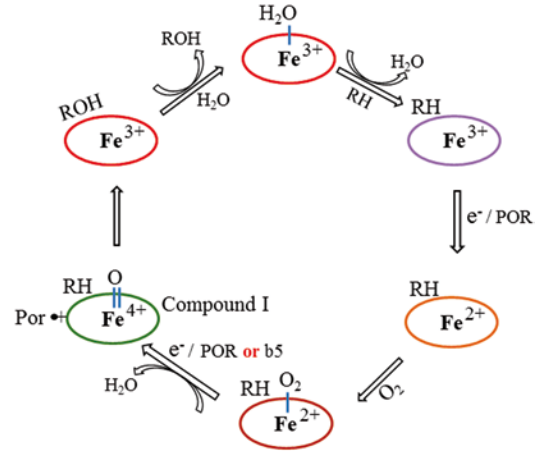

Fig. 2.9 A scheme of the P450 reaction cycle, including interactions with its redox partners, NADPH-cytochrome P450 oxidoreductase (POR) and cyt b_5. In the first step, ferric P450 binds substrate, RH. The P450-substrate complex is then reduced by POR, followed by binding of oxygen. The "second electron" is donated to the oxyferrous P450 by either POR or cyt b_5. The oxygen bond is heterolytically cleaved, resulting in the formation of compound I, the active oxidizing species. An oxygen atom is next inserted into the C–H bond of the substrate. The more hydrophilic product (ROH) dissociates from the active site. The ellipses represent the porphyrin ring while the different colors of the ring indicate spectral differences between the intermediates. $POR^{\bullet+}$ represents a porphyrin π cation radical

2.3.3 Interactions Between Cytochrome b_5 and Cytochrome P450

Bearing in mind the preceding brief background about the nature of typical interprotein interactions, the specifics of the P450-cyt b_5 interaction will be discussed. Figure 2.9 is a schematic of the reaction cycle of P450 with cyt b_5 and P450 reductase. As a result of the demonstration in hepatic microsomes: (1) that cyt b_5, which has been reduced by NADH, was partially oxidized upon addition of NADPH when substrate and oxygen were present and (2) that it coincided with product formation, it was hypothesized that cyt b_5 donated an electron to oxyferrous P450. This suggestion was consistent with two observations. One was that, under steady-state conditions in microsomes, the absorbance of oxyferrous P450 at 440 nm decreased in the presence of NADH [141]. A second observation was that NADH enhanced NADPH-supported catalysis in microsomes. Both experiments contributed support to the notion that cyt b_5 was able to provide the second electron required for P450 catalysis [148].

These reports have prompted the performance of a large number of experiments over the ensuing decades by a number of investigators in an attempt to understand how cyt b_5 enhanced catalysis in hepatic microsomes and why POR was necessary for the effect of cyt b_5. Redox potentials of ferric cytochromes P450 are ~−300 mV in the absence of substrate and are increased to ~−245 mV in the presence of substrate, while the potential of cyt b_5 is ~+25 mV (Fig. 2.9) [129, 149–152]. From a thermodynamic perspective, cyt b_5 will be unable to reduce ferric P450, but would be able to reduce oxyferrous-bound P450, which is estimated to have a potential of ~+50 mV [153]. The FMN hydroquinone of POR has an appropriate potential, ~−270 mV, to reduce the substrate-bound ferric and oxyferrous P450. This enables catalysis to proceed in the absence of cyt b_5 [33, 149]. However, the requirement for the reductase to reduce the ferric protein, thereby initiating catalysis, accounts for the observations that cyt b_5 acts after the reductase in the catalytic cycle, decreases oxyferrous P450 in hepatic microsomes, and coincides with product formation [141], implying that cyt b_5 reduces

oxyferrous P450. Employing the conditional hepatic deletion of both cyt b_5 and P450 reductase, Wolf and colleagues were able to demonstrate that cyt b_5 reductase and cyt b_5 were able to support low levels of P450 activity [140].

When purified proteins became available, it could be demonstrated that cyt b_5 could stimulate, inhibit, or have no effect on catalysis by a purified reconstituted P450 and POR [132]. Moreover, these effects were shown to depend on both the particular isozyme of microsomal P450 and the substrate. The sequence of addition of reactants to the assay mixture also influenced the results [154]. To add to the conundrum about the role of cyt b_5 in P450 catalysis, it has also been suggested that apo-cyt b_5, lacking the heme, could stimulate catalysis by selected isozymes of P450 [133, 155, 156].

To gain a better understanding of the function of cyt b_5 in P450 catalysis, its overall effect on the utilization of NADPH for product formation, rather than side product formation (superoxide and hydrogen peroxide), was investigated by several laboratories [127, 157, 158]. It was concluded that cyt b_5 enhanced coupling of NADPH utilization for product formation, i.e., the efficiency of catalysis, by decreasing the formation of the side products, hydrogen peroxide and superoxide.

With P450 2B4, cyt b_5 improved the efficiency of NADPH utilization for product formation for both poor and good substrates by approximately ~15% by generating less of the side product superoxide, which rapidly dismutates to hydrogen peroxide. These results suggest an explanation for the substrate dependent effects of cyt b_5. A 15% increase in efficiency of a poor substrate will significantly increase the absolute amount of product formation by a given amount of NADPH. In contrast, a substrate that is already metabolized with a 50% efficiency will not undergo a marked increase in the absolute amount of product formation when the reaction efficiency is simply increased by 15% [157]. Cyt b_5 lacking the C-terminus membrane-binding domain has been found by many investigators NOT to enhance P450 activity [127, 159, 160].

2.3.4 The Binding Site on P450 for Cyt b_5 and P450 Reductase

Having achieved a better understanding of the overall effect of cyt b_5 on P450 catalysis, investigators conducted experiments with the goal of elucidating the molecular mechanism by which cyt b_5 exerted its influence.

As the heme is buried and not directly accessible on the surface of type I and II P450s, it cannot accept electrons from other protein donors via direct contact between the prosthetic groups. An incoming electron must initially encounter amino acids of the P450 polypeptide [53, 161–163]. The heme is closest to the surface near the axial cysteine, which, by convention, has been designated as the proximal surface of P450. The surface closest to the heme and the cysteine has a positive potential especially in microsomal P450s ($P450_{cin}$ 869 Debye; $P450_{cam}$ 697 D; P450BM3 640 D; P450 2D6 1197 D; (http://dipole.weizmann.ac.il/) P450 17α-hydroxylase/lyase 1197 D. It is concave with the cysteine at the approximate center and bottom of the concavity. A considerable amount of evidence has accumulated from mutagenesis experiments, ionic strength manipulations, chemical cross-linking studies, crystal structures, and NMR investigations that the anionic, convex surfaces of the redox partners (cyt b_5, P450 reductase, and ferredoxins such as putidaredoxin and adrenodoxin) dock with the basic concave proximal surface of P450 [53, 128, 161, 164–168].

To recap, the interprotein interfaces are complementary with respect to the geometry and electrostatics of their interfaces, which is typical of redox protein interactions [142]. Cyt b_5 and POR are promiscuous redox proteins, capable of reducing many different proteins in both physiological and nonphysiological reactions. Thus, it is logical that the specificity of physiological reactions will be dictated by the acceptor protein. A noncognate redox partner might bind and compete with the physiological cognate donor, but if it does mediate catalysis, it usually does so at a markedly slower rate than the cognate reductase

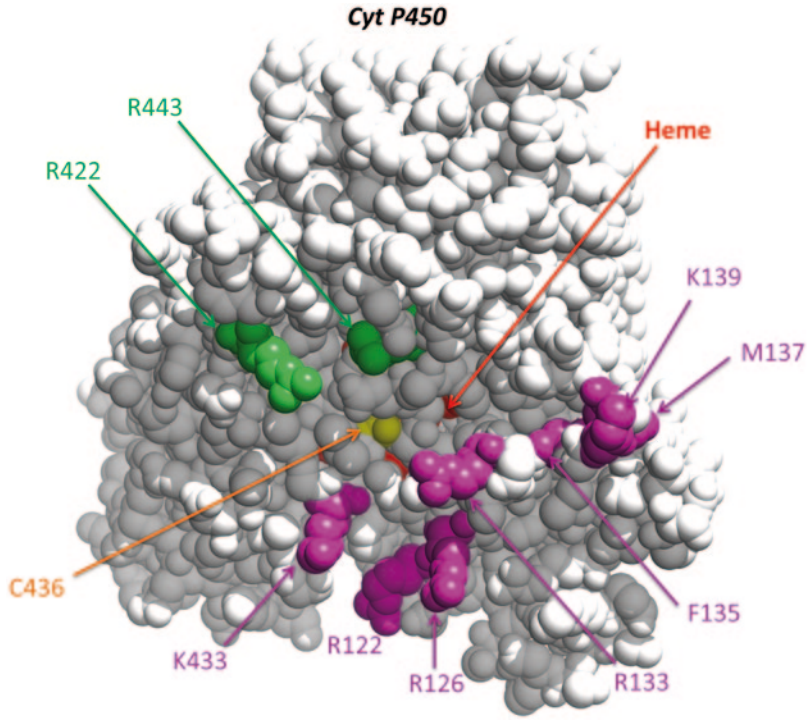

Fig. 2.10 The binding site for P450 reductase and cyt b_5 on the proximal surface of P450 2B4. Residues in *dark pink* are involved either directly or indirectly through a conformational effect in binding both the reductase and cyt b_5. These residues are in the C helix and the β-bulge. Residues in green are involved only in binding the reductase. They are located in the L helix and between the meander and the β-bulge

[168–170]. Although the microsomal redox partners will bind to the proximal surface of P450, each complex interface will be unique due to the nonidentity of each P450, but nonetheless share many characteristics.

Figure 2.10 illustrates residues on the proximal surface of P450 2B4 (1SUO) that have been demonstrated to participate in redox partner binding, either directly or indirectly, by a conformational change [53]. Residues demonstrated to participate in both cyt b_5 and P450 reductase binding are shown in dark pink, while the two residues whose mutation decreases only the affinity for P450 reductase are in green. Both basic and nonpolar residues (F135, M137) are important for the interaction. Another key conclusion from the observation of unique but overlapping binding sites for cyt b_5 and reductase is that both cannot be bound to P450 simultaneously. As a result, they will compete for binding to P450. The competition will depend on the relative abundance of each partner and its relative affinity for P450. Even though there is no evidence at this time for a protein corresponding to cyt b_5 in either *P. putida*, the source of P450cam or *B. megaterium*, the source of P450BM3, the soluble form of cyt b_5 does interact with these P450s on the proximal surface of the respective P450, albeit with significantly (2–3 orders of magnitude) decreased affinity compared to the cognate reductase [170, 171]. As predicted, anionic cyt b_5 competes with the acidic putidaredoxin for binding to P450cam [170, 172]. However, cyt b_5 does not support rapid catalysis by P450cam. In addition to binding, a specific interaction with a redox partner is required for efficient catalysis [168]. A similar situation exists with P450BM3 which is a dimeric fusion protein between a heme and diflavin P450 reductase domain [171]. Soluble housefly cyt b_5 can bind to both the separate P450BM3 heme domain and the intact protein but lacks the ability to enhance the activity of the intact pro-

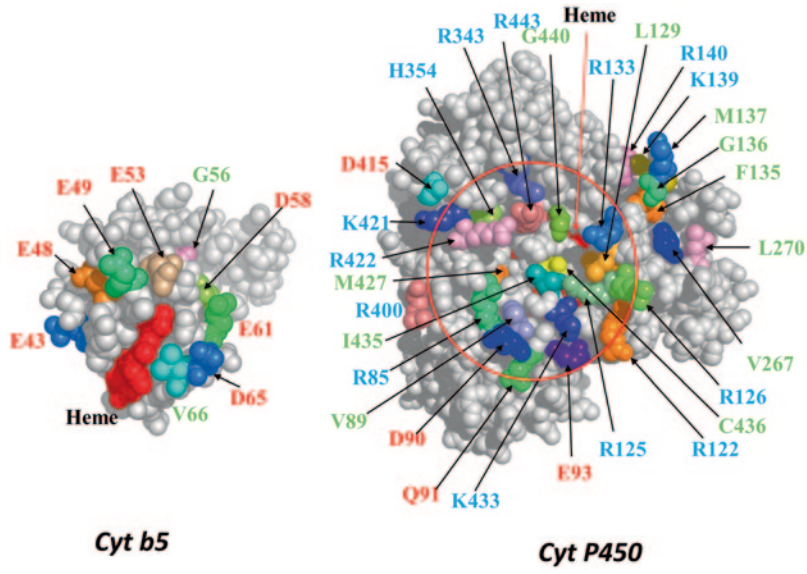

Fig. 2.11 Composite of interaction sites from different P450s with cyt b_5 and NADPH-cytochrome P450 oxidoreductase (POR). *Left*: Residues from various cyts b_5 that react with different P450s are mapped onto the surface of cyt b_5. *Right*: Residues from different P450s that react with POR are mapped onto the proximal surface of P450 2B4 (pdb 1SUO). The basic residue labels are *blue*; acidic residue labels are *red*; neutral residue labels are *green*

tein. Interestingly, intact *E. coli* flavodoxin supports a low level of enzymatic activity of P450 17A1 during expression [173].

In view of the similarity of the proximal surfaces of P450s, residues that have been implicated in the binding of either cyt b_5 or reductase by studies, either in humans or in vitro, have been mapped onto the proximal surface of P450 2B4 (pdb code 1SUO (Fig. 2.11) [174]. These residues are located in the B, C, J, K, H, and L helices, the β-bulge, and the residues between the meander and β-bulge. Data from the following P450s have been included in Fig. 2.11: CYP101, CYP102, CYP1A1, CYP1A2, CYP2A5, CYP2B1, CYP2B4, CYP2C8, CYP2C9, CYP2E1, CYP3A4, CYP6AB3, CYP17A1, CYP19. [65, 87, 134, 164, 166, 167, 169, 172, 175–185]. Figure 2.11 demonstrates that the P450s, for which there is structural information about the docking surface, all interact with their redox partners on the proximal surface as proposed [161]. Although basic residues predominate, hydrophobic residues and hydrogen bonds also contribute to the docking interface between the partners [87].

Selected proteins (CYP101, CYP1A2, CYP3A4, CYP6AB3, CYP19) appear to dock with the reductase in the B–B′ helix (residues R85, V89, D90, Q91) which is close to the substrate binding site and the I helix. Reductase binding at this site might induce conformational changes in the active site. Many of the P450 residues in the C helix, β-bulge, and N-terminus of the L helix have been demonstrated to be essential to the interprotein complex for both reductase and cyt b_5. In view of the proximity of the C helix and β-bulge to the heme, many of the residues in these secondary structures contact the heme. It is not unexpected that their residues would be important for redox partner interactions. Structural evidence (from the > 100 P450 structures in the pdb) is also accumulating that redox partner binding transmits conformational and dynamic changes to the active site and that conformational changes from the active site can be transmitted to the proximal surface. Substrate binding to

P450s typically decreases the flexibility of residues involved in substrate binding and modifies the architecture of the active site. Substrate and inhibitor binding may also modify the conformation of the redox partner-binding site. P450s are extraordinarily flexible molecules, well suited to perform their numerous functions [168, 186, 187].

Examination of Fig. 2.11 demonstrates that there is a ring of basic residues (R443, R133, R126, R125, R122, K433, R85, R422, K421, H354, R343) around the rim of the depression on the proximal surface, which is also present with some variation on the proximal surface of other P450s. The long, flexible basic residues which are components of this rim are in an excellent position to "electrostatically steer" and dock with the negatively charged surface of the redox partner to form an encounter complex (Fig. 2.6). In view of current knowledge, it appears that each P450 employs slightly different residues to react with its promiscuous redox partners. In humans, there is a single reductase that provides electrons to approximately fifty microsomal P450s and heme oxygenases, while cyt b_5 also reacts with several very different redox partners (desaturases and enzymes involved in the synthesis and biodegradation of lipids [127]. It is, therefore, necessary for the P450 to provide the specificity of the reaction. For example, three residues (Arg347, Arg358, Arg449) on the proximal surface of P450 17α-hydroxylase form a positively charged patch critical for cyt b_5 binding [160, 165]. Mutation of these residues preferentially diminished the binding and lyase activity of cyt b_5 compared to the binding and 17α-hydroxylase activity of the reductase, consistent with the notion that the P450 controls the specificity of the interaction with the redox partners [188].

2.3.5 Binding Site on Cyt b_5 for P450

The sequence of the soluble, negatively charged, heme-binding domain of microsomal cyts b_5 is highly conserved in eukaryotes with about 80% identity and very conservative substitutions. The two most conserved motifs are the HPGG, which includes one of the axial histidines, and the GXDATD/E. In mammals, the glutamate and aspartic residues are completely conserved, while Asp58 is the most highly conserved acidic residue among all the different cyts b_5 [189]. The plant heme-binding cyt b_5 domains are ~50% similar [166, 190]. Mutagenesis, cross-linking, and modeling studies indicate that anionic residues surrounding the solvent exposed cyt b_5 heme are important for binding to P450, as is a heme propionate. Most of the residues implicated in participating in binding to P450s are on or near the loops that host the two axial histidines (H44, H68), i.e., the "40s" and "60s" loops between α-helices 2 and 3 and α-helices 4 and 5, respectively [128, 134, 165, 166, 191–194]. An exception is the highly conserved Asp58 located ~14 Å away from the 60s loop [190]. Since it is located in a loop between β-strand 5 and the start of helix α-4, it may have a structural role and, as a result, may be altering the 60s loop conformation. A heme propionate has also been implicated in binding P450 (Figs. 2.12 and 2.13) [127, 128, 134]. Figure 2.11 illustrates the anionic surface of cyt b_5 with the location and identification of amino acids whose mutation has resulted in decreased interaction with a number of different P450s, respectively [127, 128, 134, 165, 166, 191–194]. Note the paucity of cyt b_5 residues deemed important for binding to P450s and that it was sometimes necessary to construct a double mutant to observe a significant decrease in function. This observation is consistent with the conclusion of Dutton and coworkers, namely that in nature interprotein electron transfer has generally been engineered to be robust and resistant to mutational changes and minor perturbations by positioning the electron donor and acceptor within 14 Å [142]. In one study, 13 residues surrounding the heme were mutated to alanine [128]. Eleven of the residues had no or only a very modest effect on the interaction with P450 2B4. Of the eleven amino acids shown not to contribute significant energy to the binding of P450 2B4, four of them (G49, V50, E53, Q54) were in contact with P450 2B4 in models of a major and minor complex between cyt b_5 and P450 2B4 (Figs. 2.12 and 2.13). Interestingly, one of the two residues observed

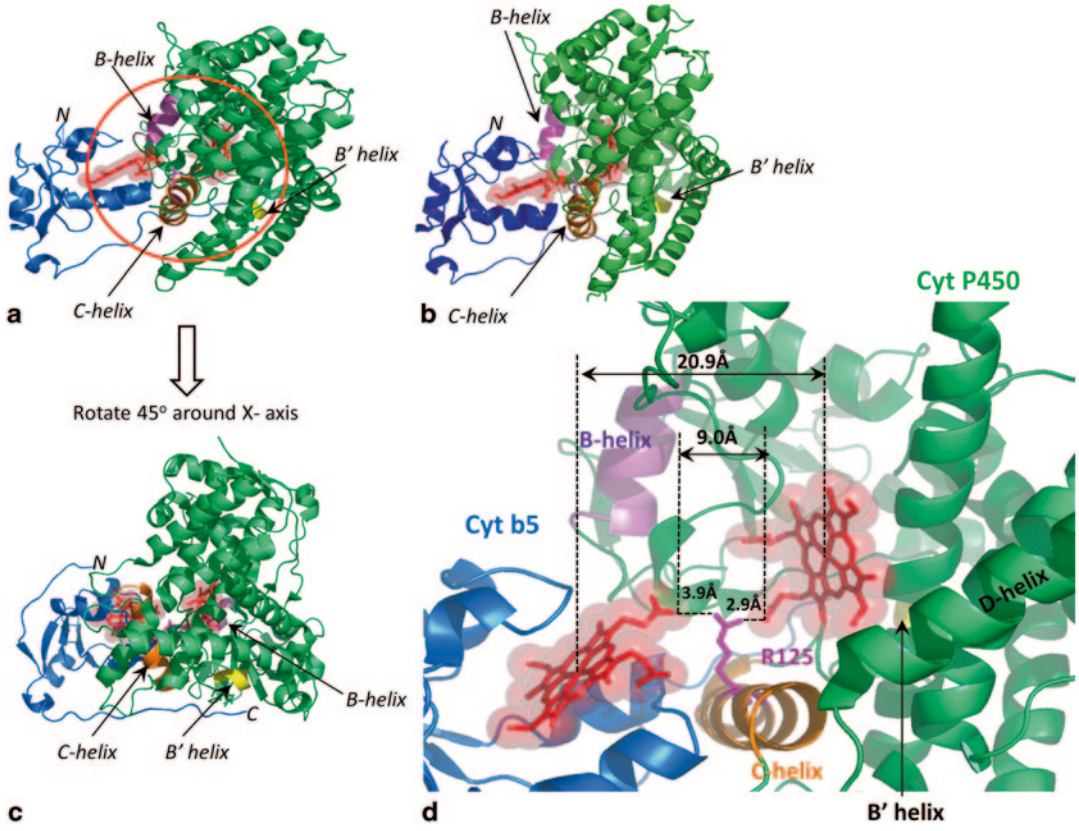

Fig. 2.12 Overview of the structure of a major and minor cyt b_5-P450 2B4 complex. Mutagenesis and NMR constraints were employed to determine the complex structures. P450 2B4 is in *green*; cyt b_5 is in *blue*; heme is in *red*; P450 Arg125 is in *magenta*. **a** The most abundant complex as determined by nuclear magnetic resonance (*NMR*). **b** The less abundant complex [128]. Note how little movement is necessary for both the 40s and 60s loops to come in contact with P450. **c** Rotation of the major complex to show the location of the terminus of the flexible linker. **d** Electron transfer predicted by HARLEM to occur between the cyt b_5 and P450 heme D propionates via P450 Arg125 [128]

to contribute most to the binding energy of the complex was hydrophobic Val66, the other was Asp65 [128].

2.3.6 Model of the P450 2B4 and Cyt b_5 Complex

On the basis of mutagenesis data from seven P450 2B4 mutants and 13 cyt b_5 mutants, a double mutant cycle analysis, and NMR-generated constraints, a model of the P450 2B4-cyt b_5 complex has been constructed using the docking algorithm HADDOCK [128]. HADDOCK first docks the two proteins as rigid bodies to minimize intermolecular energy. Next, it allows residues at the interface to move to optimize side chain and backbone orientations. Finally, the structures are refined in explicit solvent layers. Major and minor complexes were observed, indicating the dynamic nature of the complexes (Figs. 2.12 and 2.13). In the major complex, residues in the "60s loop," which flanks axial His68, are in contact with P450, whereas in the minor complex, the cyt b_5 is slightly tilted so that residues in both the "40s" and "60s" loops are in contact with P450 2B4. Altogether seventeen cyt b_5 residues were in contact with P450 2B4. Models of the cyt b_5-P450 3A4 and cyt b_5-P450 2E1 complexes, together with mutagenesis data, also indicate that the "60s loop" is likely the primary area of contact with these P450s [134, 166].

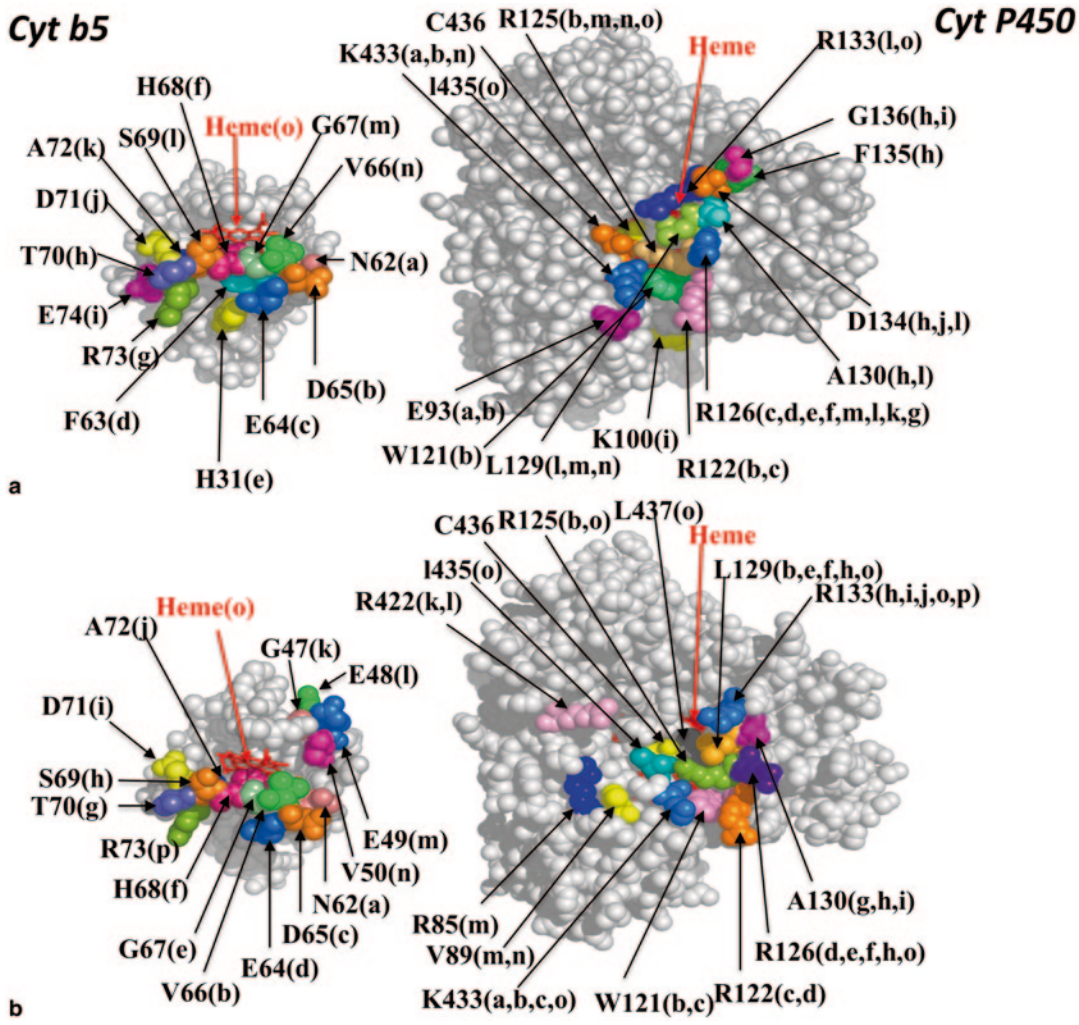

Fig. 2.13 Interface of the major and minor cyt b_5-P450 2B4 complexes. **a** The residues in contact on the interface of the most abundant, major complex. Residues on P450 that are in contact with residues on cyt b_5 are denoted with matching letters in parenthesis. For example, Arg133 (l, o) on P450 (*dark blue*) is in contact with Ser69 (l) and heme (o) on cyt b_5. Cyt b_5 is on the *left* and P450 on the *right* (pdb codes 2M33 and 1SUO respectively). Most of the cyt b_5 residues in contact with P450 are located in or near the 60s loop. Residues on cyt b_5 that are in contact with residues on P450 are denoted with matching letters in parentheses. **b** Interface of the less abundant cyt b_5-P450 complex. Note that many of the residues are the same. The most noticeable difference is that both the 40s and 60s loop of cyt b_5 are in contact with P450 [128]

Mutagenesis experiments suggest that the "40s loop" is involved in binding to P450 17A1, while P450 2C19 interacts with the residues in the "60s loop" [192].

The most notable feature of the complex is the salt bridge formed by the highly conserved Arg125 of P450 between the heme D propionates of both cyt b_5 and P450 2B4. Arg125 of P450 2B4 is homologous to $P450_{cam}$ Arg112, which has been shown to be essential for interprotein electron transfer [182]. HARLEM, an electron transfer pathways prediction program, proposed that electron transfer may occur between the heme propionates [128]. The heme edges are 9 Å apart, while the heme irons are separated by 20.9 Å, well within the generally accepted

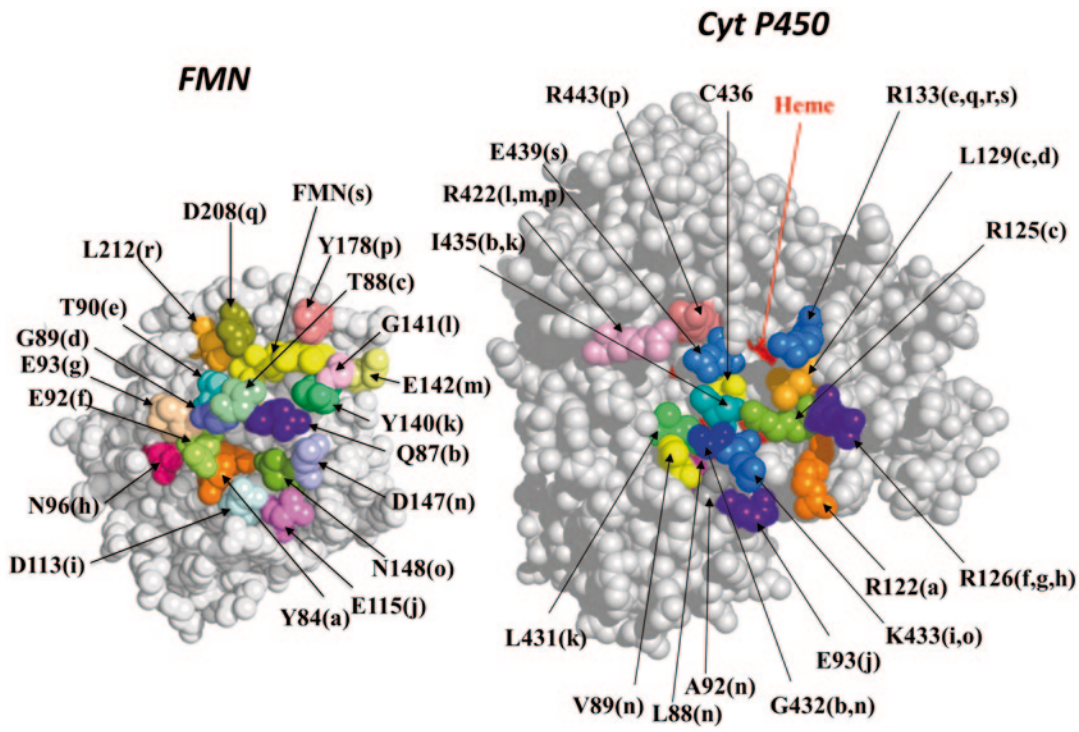

Fig. 2.14 Interface of the NADPH-cytochrome P450 oxidoreductase (POR)–flavin mononucleotide (*FMN*) domain—P450 2B4 complex. The model was generated as previously described, using mutagenesis constraints [52]. The coordinates of the FMN domain were from pdb code 3ES9 and from pdb code 1SUO for P450 2B4. Residues on the FMN domain that are in contact with P450 are denoted with matching letters in parenthesis

electron tunneling distance of 14 Å [142]. The surface area of the complex interface is ~1150 Å^2. It is formed by salt bridges, hydrogen bonds, and hydrophobic residues, as proposed for electron transfer proteins [87]. Results of a double mutant cycle analysis revealed that P450 2B4 Lys433, located three residues upstream of axial Cys436 in the β-bulge, was in contact with the acidic amino acid Asp65 and the hydrophobic Val66 of cyt b_5. Arg122 in the P450 C helix interacts with Asp65 [128]. Lysines, in CYP2E1, CYP1A2, and CYP2C9 that are homologous to Lys433 have been implicated in binding its redox partners. Due to its proximity to the heme, Lys433 and homologous lysines are well situated to transmit structural information from the redox partner to P450 and perhaps electrons.

For comparison, Fig. 2.14 shows the residues in contact in the model of the complex between P450 2B4 and the FMN domain of P450 reductase. Figure 2.5 provides an overview of the complex [53]. Interprotein contacts include salt bridges, hydrogen bonds, and van der Waals interactions. The area of the interface of the FMN domain-P450 complex is 870 Å^2, slightly smaller than the cyt b_5-P450 interface. It can be seen that P450 residues implicated in binding P450 reductase also participate in binding cyt b_5. While the different P450s all appear to utilize their proximal surface for docking, each proximal surface is unique. The interprotein complexes will be similar, but not identical, and will be formed based on the general principles of interprotein complex formation. Homologous residues may make quantitatively different contributions to the binding energy of their respective complexes. Utilization of overlapping but nonidentical sites for P450 reductase and cyt b_5 binding predicts the redox partners will compete for binding to P450 and their binding will be mutually exclusive. Experiments with P450 2B4, P450 17A1, and P450 3A4 demonstrate that cyt b_5 and P450 reductase

do, indeed, compete for docking with P450 [128, 134, 165]. In a particular situation, the relative affinity of the redox partners for P450 and the relative concentration of cyt b_5 and the reductase will determine which partner actually binds to the P450. How the binding of microsomal P450s to their partners is orchestrated in vivo is unknown. Since there are alleged to be ~5–20 molecules of P450 for every reductase molecule in microsomes, the in vivo regulation of the interprotein reaction is presumed to be highly regulated by a currently unknown mechanism. [22, 23, 195].

2.3.7 Mechanism of Action of Cyt b_5 with P450

The possible mechanisms of action of cyt b_5 with P450 have been reviewed [127]. The proposed mechanisms of action will be summarized and then discussed in light of recent experiments that have begun to provide some clarity (see Fig. 2.9 for the P450 reaction cycle). (1) One possibility is that cyt b_5 provides the second electron to oxyferrous P450 faster than POR. (2) The second possibility is that cyt b_5 enhances the utilization of NADPH for product formation, possibly because it provides the second electron faster than P450 reductase. (3) The third possibility is that P450, POR, and cyt b_5 form a ternary complex. Reductase delivers two electrons to the diheme complex via P450. Reductase then dissociates from the ferrous diheme complex. After oxygen binds to P450, the ferrous cyt b_5 immediately reduces oxyferrous P450. It was proposed that reduction of oxyferrous P450 by bound cyt b_5 would occur faster than reductase dissociation to retrieve a second electron. (4) The fourth possibility is that cyt b_5 acts as an effector in the reaction with P450.

1. When the rates of reduction of an oxyferrous microsomal P450 by cyt b_5 and P450 reductase were directly measured and compared, it was observed that cyt b_5 and reductase both reduced oxyferrous P450 2B4 at the same rate [149]. Unexpectedly, the P450 reacted differently following reduction, depending on whether it had accepted an electron from cyt b_5 or POR. Presumably this occurs because each redox partner elicited a different conformational change in the active site on the distal side of the heme. In the presence of cyt b_5, product was formed rapidly with the substrate benzphetamine and with ~52% coupling, whereas product formation was significantly slower (~10–100-fold) and less coupled (~32%) with the reductase. Coupling refers to utilization of electron equivalents for product formation. How much slower depends on the substrate [196]. How generalizable a phenomenon and observation this is awaits the results with different P450 isozymes.
2. Numerous investigators have indeed shown that cyt b_5 may enhance the coupling of NADPH utilization for product formation at the expense of side product (hydrogen peroxide and superoxide) formation [157, 158, 197]. While this is a reproducible observation, it is not a molecular explanation for the actions of cyt b_5. Increased efficiency of NADPH utilization could be explained by the more rapid rate of product formation, which allows less time for production of the side products hydrogen peroxide and superoxide.
3. While it was established as early as the 1980s that ferrous P450 could reduce ferric cyt b_5, the role of such a reaction in altering the activity in a reconstituted system is uncertain [158, 198, 199]. Moreover, the alleged formation of a ternary complex between P450, cyt b_5, and P450 reductase has been challenged [57, 200]. A functional ternary complex is also incompatible with the large amount of mutagenesis data that reveals cyt b_5 and the reductase have overlapping binding sites on the proximal surface of P450s and with observations from several laboratories on purified reconstituted systems that the redox partners compete with one another for a binding site on P450 [134, 139, 165].
4. There is a significant amount of evidence from a number of laboratories that cyt b_5 can act as an effector for some P450s. P450 2D6 and P450 1A1 are known exceptions [201]. One of the earliest and most convincing examples is the partial conversion of the hexacoordinate

low-spin to the pentacoordinate high-spin heme iron, which occurs when cyt b_5 binds to a P450 (2B4, 3A4, 17A1, 4A7). Displacement of the sixth axial ligand, water, from the heme of P450 by cyt b_5 in many instances is greater when substrate is present in the active site. One of the simplest explanations for the displacement of the water from the P450 heme iron is a conformational change induced by the binding of cyt b_5 that is subsequently transmitted to the active site, resulting in the displacement of water from the iron. In view of the tremendous flexibility of P450s exhibited by the P450 atomic resolution crystal structures, there are several plausible pathways through which conformational changes could be propagated from the proximal surface to the distal substrate-binding pocket. One is that docking with residues on the C helix can transmit changes via the substrate-binding B-B′ loop and helices to the I helix near the conserved active site threonine and acidic residue.

Of the four previously proposed mechanisms of action of cyt b_5, it appears that there is insufficient evidence for ternary complex formation and a more rapid reduction of oxyferrous P450 by cyt b_5 compared to POR. Both reduce P450 at the same rate. It is proposed that cyt b_5 simultaneously has two effects on the P450 isozymes it stimulates. Its interaction with P450 results in both electron donation to the oxyferrous protein and a substrate- and isozyme-dependent conformational change in the active site that allows catalysis to occur more rapidly. One possibility is that the active site conformational change induced by cyt b_5 leads to the more rapid formation of the active oxygenating species, compound I, compared to the reductase [158, 196]. The more rapid turnover in the presence of cyt b_5 results in less time for side product formation, which in turn increases the coupling of NADPH consumption to product formation.

2.3.8 Apo Cytochrome b_5

Currently, there is no consensus about whether apo cyt b_5 (cyt b_5 devoid of heme) is able to act only allosterically to stimulate catalysis by P450 or whether apo cyt b_5 must first bind heme to form holo cyt b_5, which is both capable of electron transfer and an allosteric effect. One of the difficulties in analyzing the literature about apo cyt b_5 is that dissimilar conditions have been employed to investigate not only different isozymes but also identical proteins, precluding a satisfying conclusion about the effects of apo cyt b_5 on P450 catalysis. NMR studies have shown that the structure of apo cyt b_5 and cyt b_5 are similar, with only minimal differences in their secondary structure [202]. As a result, they are expected to have a similar interaction with P450s. Models of a complex between P450 3A4 and apo cyt b_5 and holo cyt b_5 have been constructed. They indicate that both complexes form very similar docking sites on the proximal surface of P450 3A4 in a location that overlaps with the POR binding site [134].

Apo cyt b_5 has been found to stimulate some P450s (P450 3A4, the 17,20-lyase reaction of P450 17A1, and P450s 2A6, 2C8, 2C9, 2C19, 3A5, 4A4, 4A7 and 6A1) [133, 134, 155, 156, 158, 197, 201], but not others (P450 2B4, 2E1, and 2D6) [132, 133, 137, 203]. Apo cyt b_5 can only stimulate a P450 activity if the holo cyt b_5 can enhance the activity. Apo cyt b_5 has also been reported to induce a spin-state change in some P450s, which indicates that apo cyt b_5 binds to P450. This is not surprising in view of their similar structures [134].

Recently, the allosteric stimulatory effector role of apo cyt b_5 was challenged [204, 205]. It was proposed that the stimulatory effect of apo cyt b_5 was due to the transfer of heme from P450 3A4 and P450 17A1 to apo cyt b_5, thereby creating holo cyt b_5, which is known to possess stimulatory properties. A more compelling argument about the lack of the stimulatory ability of apo cyt b_5 was their demonstration that neither a redox inactive Zn-substituted protoporphyrin IX derivative of cyt b_5 nor an axial His67Ala mutant that is unable to bind heme was able to stimulate the activity of either P450 3A4 or P450 17A1. Moreover, the addition of a heme scavenger, apo myoglobin, to the reaction mixture eliminated the stimulatory effects of the apo cyt b_5.

These studies prompted a reexamination of the stimulatory effects of apo cyt b_5 in a reconstituted system with P450 3A4 and 17A1 [133]. The reexamination concluded that far less heme transfer occurred than could be accounted for by the stimulatory effects of apo cyt b_5. Furthermore, apo myoglobin did not inhibit the stimulatory effects of apo cyt b_5. The reexamination did not include investigation of the effects of redox inactive cyt b_5 which had been reconstituted with a Zn-substituted protoporphyrin IX. Nor did it investigate whether cyt b_5 mutants that were unable to bind heme were still stimulatory.

Several laboratories have reported that cyt b_5 reconstituted with Mn protoporphyrin IX, which is redox inactive in the reconstituted system, was unable to stimulate the activity of P450 [132, 139, 200, 206, 207]. In fact, as the concentration of Mn cyt b_5 was increased relative to a constant amount of P450 and P450 reductase, NADPH consumption and activity decreased and the rate of reduction of ferric P450 was diminished. These effects of Mn cyt b_5 are consistent with the ability of Mn cyt b_5 to decrease the rate of reduction of ferric P450 by competing with P450 reductase for binding to P450 [139]. In addition, it has been reported that siblings with a homozygous axial histidine variant of cyt b_5, His44Leu, exhibited a phenotype with abnormal genitalia and low androgens, indicative of an apparently isolated deficiency of the cyt b_5 requiring 17,20-lyase activity of P450 17A1. An elevated methemoglobin (Fe^{+3} Hb) was also noted. This human phenotype is supportive of a nonfunctional apo cyt b_5 in vivo [208]. Drug metabolism was not investigated in these individuals.

In conclusion, in spite of the different isozymes and experimental conditions involved, apo cyt b_5 does appear to affect the activity of selected P450s. Its mechanism of action continues to be vigorously debated. Nevertheless, the weight of the evidence is pointing to the likelihood that only P450s that are able to transfer their heme to apo cyt b_5 to form holo cyt b_5 and also have activities that are increased by holo cyt b_5 are stimulated. For example, P450 3A4 and 17A1 have been observed to transfer heme to apo cyt b_5 under experimental conditions, whereas P450 2B4 does not significantly transfer heme to apo cyt b_5. Due to the similarity of apo- and holo cyt b_5 structures, apo cyt b_5 may also compete with reductase for a docking site on P450, which, depending on the molar ratios of the redox partners to P450 and their relative affinities for P450, could decrease the activity of the isozyme even in the absence of holo cyt b_5 formation.

2.3.9 Summary of Mechanism of Action of Cyt b_5 on P450

Although our understanding of how cyt b_5 can increase, decrease, or have no effect on catalysis by P450, and why its actions are dependent on the isozyme and substrate, is still incomplete, significant progress has been made in the past four decades in elucidating its mechanism of action. The fact that both cyt b_5 and reductase reduce oxyferrous P450 at the same rate indicates that the mechanism of action of cyt b_5 occurs after reduction of oxyferrous P450 in the reaction cycle. Its stimulatory effects are consistent with an ability to generate the active oxidizing oxyferryl species, compound I, more rapidly than P450 reductase. It is likely that this occurs by inducing a conformational change in the proton delivery network in the P450 active site. More rapid formation of compound I would allow less time for side product formation and result in increased efficiency of catalysis.

Evidence is also accumulating that is supportive of the notion that cyt b_5 and reductase compete for a binding site on the basic proximal surface of P450s. The ability of a redox partner to bind a P450 will depend on the relative concentrations and relative affinities of the redox partners for the specific P450 isozyme. At higher molar ratios compared to a constant P450:POR 1:1 ratio, cyt b_5 will abort the reaction cycle by preventing P450 reductase from reducing ferric P450, while at lower molar ratios cyt b_5 is stimulatory [196]. No effect is observed when the opposite effects cancel. The actions of cyt b_5 on different isozymes of P450 is inferred to depend on its ability to induce the conformational changes in the active site necessary for more rapid catalysis and on

its affinity for the particular P450 in comparison to the POR.

A final dilemma is: why does the effect of cyt b_5 vary with the substrate even when it is being metabolized by the same P450? This is the least understood, most enigmatic of the effects of cyt b_5. It has been observed that under similar conditions, the same P450 utilizes qualitatively the same amount of NADPH regardless of the substrate, while cyt b_5 increases the efficiency of catalysis by roughly 15% regardless of the substrate [157]. These results lead to the speculation that a poor substrate whose metabolism is 2% coupled will have the absolute amount of its metabolism increased by ~ seven times. In contrast, a good substrate whose metabolism is approximately 50% coupled will have the absolute value of its metabolism enhanced by merely 30%, which may be within experimental error [157]. How generalizable this speculation is awaits detailed studies of other P450s and substrates. Both reactions should be subject to inhibition by high concentrations of cyt b_5. If cyt b_5 really does increase the efficiency of catalysis by approximately the same amount irrespective of the substrate, it implies that its putative allosteric effect is probably not always dependent on the substrate. The highly flexible nature of P450s has been noted and likely contributes to the variety of results.

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Activation of Molecular Oxygen in Cytochromes P450

3

Ilia G. Denisov and Stephen G. Sligar

3.1 A Brief History of "Oxygen Activation"

The cytochrome P450s have been the focus of attention for legions of investigators. For the basic scientists, the unique spectral properties of this heme protein provided fascinating challenges for the bioinorganic chemist. The difficult chemistry of adding an oxygen atom to an unactivated alkane intrigued the bioorganic chemist, and the need for electron transfer with proton involvement brought the physical biochemists to the table. With the known processes of the archetypical heme proteins myoglobin and hemoglobin, as well as the reductive chemistry operating in the cytochrome oxidases, it was no surprise that investigators from these fields were amongst the first to focus their attention on cytochrome P450 and its redox partners. The concept of "oxygen activation" thus comes from two directions. First, although atmospheric dioxygen can be reactive at room temperature, e.g., in the formation of rust, typical hydrocarbons are stable until combustion at elevated temperatures. Thus, facile hydrocarbon hydroxylation or epoxidation near 37 °C requires enzymatic "activation." From the protein standpoint, the reversible binding and release of atmospheric dioxygen by hemoglobin led to documentation of an intermediate state—the ferrous heme—O_2 complex. Nature evolved the protoporphyrin IX prosthetic group within the globins to protect this oxy-ferrous complex, resulting in a relatively stable species, although after long time intervals this intermediate would "auto-oxidize" releasing superoxide and converting the heme iron to the ferric state. Since it was realized early on [1] that the cytochrome P450s also contained protoporphyrin IX heme as a prosthetic group, and hence could bind atmospheric dioxygen, the protein must be doing something to "activate" the bound dioxygen for catalysis.

The canonical overall reaction of cytochrome P450 involves the reductive scission of the O–O bond of atmospheric dioxygen to release a single molecule of water with the transfer of a single oxygen atom to the substrate:

$$S + O_2 + 2_{e-} + 2H+ \rightarrow H_2O + S - O$$

Cytochrome P450s are thus "oxygenases" as one or more oxygen atoms from O_2 are incorporated into a substrate molecule, following the discovery of this class of enzymes by Hayaishi and Mason in 1955 [2, 3]. Very soon thereafter the first experimental proof of steroid hydroxylation by a mammalian oxygenase was identified by using $^{18}O_2$ for the reaction [4], although the enzyme responsible for this, CYP11B1, was not identified until 1965 [5]. A beautiful review

I. G. Denisov (✉) · S. G. Sligar
Department of Biochemistry, The School of Molecular and Cellular Biology, University of Illinois, 116 Morrill Hall, 505 South Goodwin Avenue, Urbana, IL 61801, USA
e-mail: denisov@illinois.edu

S. G. Sligar
e-mail: s-sligar@Illinois.edu

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of the early P450 history was provided by Estabrook [6]. Since a single oxygen atom is inserted into a substrate, the P450s are "monoxygenases" and require additional redox transfer partners to provide the two electrons (and potentially the two protons) necessary to reduce the other oxygen atom from O_2 to water. Historically, this led to the cytochrome P450s also being called "mixed function oxidases" as they operated like a half-way point of the cytochrome oxidase stoichiometry in which four electrons and protons are used to fully convert O_2 to two molecules of water. As we now know, the cytochromes P450 can carry out a variety of additional organic transformations, including carbon–carbon bond scission and formation, dealkylation, heteroatom oxygenation, and halogenation/dehalogenation. We will discuss these other reactivities of the cytochromes P450 later in the chapter in the context of what they teach us about the various states of "oxygen activation." Since this chapter is devoted to the mechanisms of oxygen activation, it is useful to briefly mention early ideas of how the relatively inert O_2 molecule could be "activated." Again we can organize the discussion along two lines of focus: the enzymology of the P450 hemoprotein and the dioxygen molecule itself.

Debates as to the mechanisms of oxygen activation heated up in the early 1970s. From the standpoint of O_2, this was also the era of intense arguments as to the chemical reactivity of superoxide, a one electron reduced O_2. Early discussion by Fridovich and others [7] suggested O_2^- itself could attack unactivated carbon centers, while others, led by Fee et al. [8], argued that superoxide was at best a mild reductant and could not by itself institute carbon oxidation. At the same time in history, enzymologists documented the existence of the ferrous dioxygen complex of P450 isolated from *Pseudomonas putida* (P450 CYP101A1). Since this protein could be obtained in large quantities, it could be investigated by a plethora of spectroscopies. Using Mössbauer spectroscopy it was shown that in the ferrous-dioxygen intermediate of CYP101A1, stabilized at cryogenic temperatures, the iron was in the ferric state, analogous to the Weiss model proposed for hemoglobin and myoglobin. If the iron looks ferric and there is an extra electron in the $Fe–O_2$ system, then the electron density must favor the superoxide resonance form. Hence the thought: Was the active form of O_2 in P450 catalysis the superoxide anion? Reality set in, however, when it was noted that the $Fe–O_2$ complex of heme proteins could not carry out even simple oxygenation reactions—a second electron was required for "activation."

The early 1970s was also the time when interesting chemistries of the second-row nonmetals carbon and nitrogen were revealed when they were missing two electrons from their valence shell. These so-called carbene and nitrene species were shown to be able to directly insert into C–C, C–H and other organic bonds. What about oxygen? Could a six-electron oxygen atom provide the observed reactivity of the P450 enzymes? The term "oxene transferase" was proposed by Ullrich and coworkers to describe this form of activated oxygen [9]. Simple electron counting from a ferrous–dioxygen complex after a second electron input and the release of water indicated the presence of a six-electron oxygen atom somehow bound to a ferric heme. On the other hand, it was difficult to see how such an electron-deficient species could dissociate from the heme and react with a nearby substrate.

The solution to the identification of the "active oxygen species" in P450 catalysis came in 1976 through the efforts of Groves in collaboration with the Coon laboratory [10, 11]. Understanding the nature of an "oxene" bound to ferric heme, Groves realized that there would be two open orbitals on the oxygen that could initiate radical chemistry. He proposed an "oxygen rebound" mechanism wherein this species, formally at the redox state of compound I as observed in the peroxidase class of enzymes, could abstract a hydrogen from a substrate C–H bond, formally generating a hydroxyl radical bound to heme that could then undergo radical recombination with the substrate carbon radical to generate the hydroxylated product. Showing the transient existence of a substrate carbon radical intermediate was strong evidence for this being the intermediate in oxygen activation [11, 12].

3.2 The Plethora of Chemical Reactivities of Cytochrome P450

The initial focus on the ability of P450 to catalyze the oxidation of an unactivated carbon center was a driver for the chemical community, while parallel interests that focused on metabolic transformations in humans expanded the spectrum of activities associated with P450 metabolism. In addition to hydroxylation of unactivated alkanes, this includes epoxidation of olefinic substrates, the addition of oxygen to heteroatoms such as sulfur, the dealkylation of amines, and the formation and breakage of carbon–carbon bonds. These include reactions involved in human health and disease, such as the epoxidation of aromatics as part of carcinogen activation (e.g., benzo(a)pyrene) and facile heteroatom dealkylation as exemplified by the *O*-demethylation that converts codeine into morphine. These human relevancies brought the large body of pharmacologists and toxicologists into the community studying the cytochromes P450. With the growing involvement of P450 in multiple biotransformations, a natural question emerged as to the number of isozymes that might be present. Initially, several variants were found in animal liver, a key site for first pass metabolism. They were first isolated as pure proteins though enormous efforts by the Coon laboratory and others, with the isozymes labeled LM1, LM2, LM3, LM4, etc., for liver microsomal fraction 1, etc. The general feeling at the time was that there could be a dozen or even 20 different isozymes of P450 in animals and perhaps a few more in bacteria and plants. As is beautifully described elsewhere in this volume, there are now over 20,000 P450 genes identified [13]! The functions of all these P450s can be artificially separated into two classes: Those involved in the synthesis of intermediary metabolites, such as prostaglandins and hormones in humans, and those involved in catabolic reactions often associated with xenobiotic breakdown—most prevalent in the human liver, kidney, and epithelial tissues. This classification also applies to plants, insects, etc., as described by Schuler et al. in this volume (Chap. 7).

3.3 The Three-Dimensional Structure of Cytochrome P450

By the mid-1970s enzymologists were comforted by the availability of a three-dimensional structure of their enzyme, although the technology was primitive by today's standards. One requirement to obtain an X-ray structure in this era was for a substantial amount of highly purified protein. The only P450 available in the needed quantity and quality was P450cam or CYP101A1. The crystallization and solution of the structure is told by Poulos and Johnson in this volume together with those of several other soluble P450s. The vast majority of the P450s in nature, however, are anchored to a membrane and the solution of membrane protein structure remains a significant hurdle today. Indeed, one entire session of an international P450 meeting was devoted to the debate as to how good a structural model CYP101A1 would be for the membrane-bound P450s [14]. Johnson and Poulos (Chap. 1 in this volume) summarize the amazing progress in solving the structure of the membrane-bound P450s. We now recognize that all members of this large super family of P450s possess basically the same fold, with subtle differences being present that reflect specificity for substrates and redox partners. Additionally, the past four decades of work have unambiguously shown that all P450s operate by basically the same reaction cycle (Fig. 3.1), including the stoichiometry of oxygen and reducing equivalents. However, the degree of coupling, or efficiency of converting atmospheric O_2 and electrons to substrate-derived products can vary widely.

Most P450s operate with a single substrate-binding site, often with the high degree of specificity needed, for example, in hormone biosynthesis. However, some P450s can bind more than one substrate molecule, either in an enlarged active site or in a distant effector or allosteric site. This can lead to a profound effect on metabolic throughput as will be discussed in detail subsequently.

The remainder of this chapter, as well as contributions from other authors, will address the spectroscopic characterization of the intermedi-

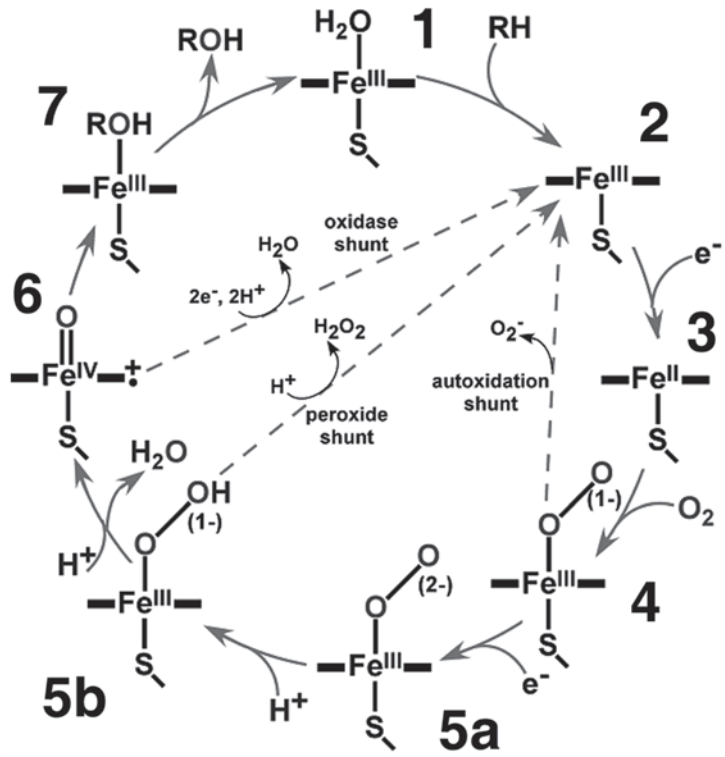

Fig. 3.1 Reaction cycle of cytochrome P450, reproduced with permission from American Chemical Society from [15]

ate states that lead to the ultimate oxygenating species operating in the cytochromes P450. We will also address the aspects of the protein structure that allow control of electron and proton input into the catalytic cycle to control the stability and reactivity of these intermediate states. Appropriate results that define the side uncoupling pathways, as well as other forms of the reduced oxygen-bound P450 heme that have the potential for substrate metabolism, will conclude this review and also provide the critical link to other forms of "active oxygen."

3.4 Substrate Binding, Spin Shift, and Redox Potentials

Substrate binding to cytochrome P450 is an important step in the overall mechanism of P450 catalysis, not only because it is necessary to position the substrate in the proper orientation in the immediate vicinity of the heme bound catalytically competent "active oxygen." Equally important, it serves as the trigger activating the electron transfer from the redox partner to the heme iron resulting in reduction of the iron from the ferric Fe^{3+} to the ferrous Fe^{2+} state. This, in turn, is necessary for binding of oxygen to the ferrous cytochrome P450 and formation of the oxygenated intermediate. In general, the regulatory role of substrate binding as the trigger initiating the reduction is used in the cytochromes P450 [16, 17], as well as in some nonheme enzymes [18], to minimize production of reactive oxygen species and unproductive waste of nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH).

Usually much tighter substrate binding is observed for P450s involved in specific biosynthesis of hormones and other regulatory compounds. Examples include the high affinity of cytochromes P450 involved in steroid hormone biosynthesis towards their natural substrates and other synthetic steroid compounds [19–21]. Interestingly, many P450s that formally belong to this class can also bind and metabolize compounds not related to their native substrates, albeit with lower affinity and efficiency. Such examples

are described for CYP101A1 [22–24], CYP102 [25–27], and CYP46 [28]. For xenobiotic metabolizing cytochromes P450, which can bind and catalyze oxidative transformations of various organic molecules with a very broad distribution of chemical structures and molecular masses, lower substrate affinities with dissociation constants in the range of 10^{-5} to 10^{-3} M are more typical. Apparently, weaker substrate binding is the price for their broad substrate specificity, a requirement for the first line of chemical defense of the organism against myriads of alien, potentially toxic, and dangerous compounds. Multiple examples are described in comprehensive reviews [29–31].

The binding of hydrophobic substrates usually leads to displacement of water from the substrate-binding pocket, including the water molecule coordinated to the heme iron as the sixth (axial) ligand, as shown in Fig. 3.2 for CYP101A1. The transition of the ferric iron atom Fe^{3+} from the hexacoordinated to the pentacoordinated state results in a spin-state transition from low spin (S = 1/2) to high spin (S = 5/2). This change in the coordination state of the heme iron gives rise to an upshift of redox potential, which is essential for efficient reduction of the enzyme from the ferric to the ferrous state.

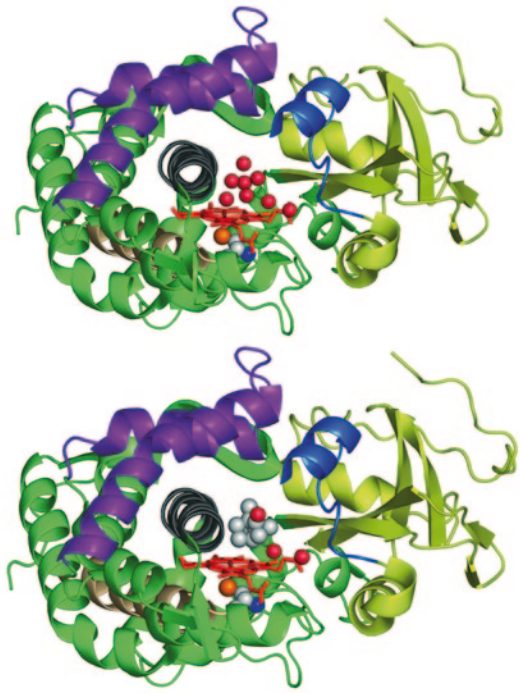

Fig. 3.2 X-ray structures of CYP101A1 without substrate (1PHC.pdb [32], *top*) and with the substrate camphor (2CPP.pdb [33], *bottom*). Shown are also the water molecules (*red* spheres) occupying the substrate-binding pocket, one of them coordinating to the heme iron as the sixth ligand

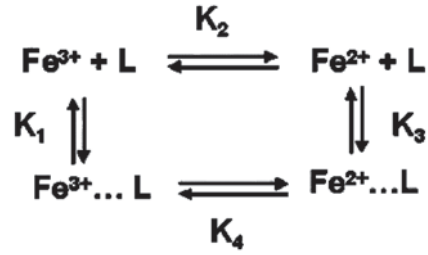

Fig. 3.3 Coupling of the ligand L binding equilibria to the ferric and ferrous heme protein with binding constants K_1 and K_3 and of redox equilibria in the substrate free or substrate bound protein with equilibrium constants K_2 and K_4

The difference in redox potentials between pentacoordinated and hexacoordinated porphyrins can be illustrated using the thermodynamic cycle shown in Fig. 3.3:

Here the ligand **L** can bind to the heme iron with binding constants K_1 and K_3, which are different for the ferric and ferrous states, while K_2 and K_4 define the redox equilibria for the five-coordinated high-spin and six-coordinated low-spin heme iron respectively [16]. The overall redox equilibrium between Fe^{3+} and Fe^{2+}, i.e., the midpoint potential, can be shifted towards the strong binder, if the ligand is present [34]. In the aqueous solution, water or a hydroxide always favors the ferric state as compared to ferrous, so that $K_1 > 1$ and $K_3 < 1$, and Fe^{3+} is typically six-coordinated in cytochromes P450 in the absence of a substrate, while Fe^{2+} is five-coordinated. As a result, the thermodynamic (redox) equilibrium between the ferric and ferrous states of the heme iron is shifted to the former in the absence of substrates, while substrate binding displaces the water molecule from the sixth coordination position, thus destabilizing the ferric state and lifting the midpoint potential. Experimentally measured shifts of the redox potentials in cytochromes P450 caused by substrate binding are in the range 80–

170 mV [16, 35–37]. In most cases, cytochromes P450 saturated with substrates are reduced much faster [37–41]. Acceleration of the first electron transfer to cytochromes P450 in the presence of a bound substrate represents an important thermodynamic regulatory mechanism, preventing futile consumption of redox equivalents and formation of toxic superoxide and peroxide, as will be discussed in a subsequent section of this chapter. In addition, Marcus theory analysis suggests a faster electron transfer in the presence of substrate due to a lower reorganization energy [42]. The spin-state equilibrium in cytochromes P450 is temperature dependent and can be probed by temperature jump studies. Direct kinetic measurements show that the typical rates of spin-state relaxation after temperature jump are in the range of 400–2000 s^{-1} for CYP101A1 [43] and 800–2500 s^{-1} for CYP102A1 [44]. The same thermodynamic coupling is responsible for the higher affinity of cytochromes P450 with respect to hydrophobic substrates at higher temperatures that is observed experimentally [44].

Substrate binding is usually fast for the soluble P450s, with apparent rates of 10^2 to 10^3 s^{-1} [45] and second-order rates ~10^6 to 10^7 $M^{-1}s^{-1}$ [45, 46]. This fast binding and simple 1:1 stoichiometry is usually observed for the efficient bacterial P450s with their natural substrates, i.e., CYP101A1 with camphor [46, 47]. Comparison of camphor binding and dissociation kinetics with mutants generated to perturb the equilibrium-binding constant demonstrated fast binding in all cases, with the affinity exclusively dependent on the dissociation rate [24]. For instance, the T101M mutant had the same camphor-binding rate as the wild-type enzyme, $k_{on} = 3 \times 10^7$ $M^{-1}s^{-1}$, but an almost tenfold higher dissociation rate $k_{off} = 192$ s^{-1}. Fast substrate binding was also reported for many other cytochromes P450, such as CYP102A1 [48] and other soluble bacterial enzymes. In many cases purified and solubilized eukaryotic cytochromes also show fast substrate binding [49]. However, in some cases, very slow substrate-binding kinetics have been observed, such as those reported for cholesterol derivatives binding to P450scc in lipid vesicles, where type I spectral changes were monitored on the scale of 15 min and apparent first-order rates obtained in the range of (4–9) 10^{-4} s^{-1} [50]. Such results are probably due to the extremely low solubility of cholesterol and its derivatives and slow redistribution between the aqueous phase and lipid bilayers [19]. The kinetics of NAD(P)H-dependent reduction of cytochromes P450 in the presence of their redox partners almost always strongly depends on the presence of their substrates. Exceptions from this general rule are reported for several cytochromes P450 that are predominantly in the high-spin ferric state even before addition of a substrate, such as CYP1A2 [51–53]. These observations are in line with the redox thermodynamics modulated by substrate binding described above (Fig. 3.3). Typically, reduction of substrate-free P450 enzymes is very slow with apparent rates in the range of 10^{-4}–10^{-2} s^{-1} [54], and is much faster (sometimes by several orders of magnitude) in the substrate-bound state [17]. Sometimes the first electron-transfer step is identified as the rate-limiting step, as shown for CYP7A1 [55]. The significant acceleration of P450 reduction in the presence of substrates is easily seen in the steady-state kinetics of NAD(P)H consumption, as reported for both bacterial and eukaryotic cytochromes [56]. The acceleration of NAD(P)H oxidation can be used as an empirical test for the screening of new compounds as potential substrates for a given cytochrome P450 [57, 58] or as a rough measure of P450 activity [59].

Interactions with redox partners are not only necessary to bring the electron donor close to the heme for efficient electron transfer. Recent structural studies of the complex of CYP101A1 with its natural redox partner, the iron–sulfur protein putidaredoxin (Pdx) [60, 61], confirmed the important allosteric regulatory role of these interactions that was first suggested in 1974 [62]. Perturbations of the CYP101A1 heme environment when complexed with its redox partner Pdx have been detected using various spectroscopic methods [63–67]. Early work by Davies and Sligar demonstrated the redox-dependent affinities of Pdx and P450 and the critical residues involved [68]. What was missing, however, was a linkage between structure and the functional implications

caused by Pdx binding. It was the X-ray structure of the complex [60, 61] that clearly demonstrated that Pdx binding results in opening of the cleft in the I-helix that is necessary for directed proton delivery to the coordinated dioxygen. Thus, interactions with oxidized Pdx favor the open conformational state of CYP101A1 [60, 61]. However, Goodin et al. demonstrated an opposite effect of reduced Pdx binding on the conformational state of CYP101A1 [69]. Taken together, these results reveal a sophisticated pattern of allosteric regulatory effects of Pdx on the structure, dynamics, and functional properties of CYP101A1. In the first step, binding of reduced Pdx stabilizes the substrate-bound closed state of ferric CYP101A1 and provides optimal conditions for the first electron transfer. After reduction of the heme, oxidized Pdx dissociates and oxygen binds to the heme iron atom. Binding of reduced Pdx to the oxy-complex stabilizes the latter against autoxidation, preventing the heme from autoxidation and resulting in transfer of the second electron and formation of the peroxo-ferric intermediate. Finally, bound oxidized Pdx favors the open conformational state of CYP101A1, with the functionally important rearrangement of residues Asp251 and Thr252 in the I-helix that are necessary for efficient proton delivery to the dioxygen moiety of the peroxo-intermediate and formation of compound I (see Chap. 1 by Poulos and Johnson). Allosteric effects of interactions with redox partners have also been suggested in other systems. Fusion with different redox partners changed the regiospecificity of catalysis and also the range of chemical transformations catalyzed by the multipurpose cytochrome P450 MycG [70].

3.5 Oxygen Binding and the Structure of the Ferrous Dioxygen Complex

The binding of dioxygen to ferrous cytochrome P450 leads to formation of the oxy-complex, which is the last relatively stable intermediate in the catalytic cycle. This complex has dioxygen coordinated end-on to the heme iron with partial transfer of electron density from the iron to the dioxygen moiety. Based on spectroscopic and structural data [71–75], the latter can be described as partially superoxide. In general, most properties of the oxy-complexes in cytochromes P450 are similar to those of other heme proteins, including the myoglobins, hemoglobins, and heme oxygenases. An overview of the structural studies of oxy-complexes in various heme enzymes was published in 2007 [76]. Oxygen binding to cytochromes P450 is usually fast and not rate limiting for P450 catalysis at ambient conditions. Kinetic studies show second-order binding rates for CYP101A1 in the range of (0.8–1.7) 10^6 M^{-1} s^{-1} at 4–25 °C in the presence of camphor [77, 78]. These rates correspond to apparent first-order binding rates 200–300 s^{-1} in aerated solutions. Similar rates have been reported for CYP1A2 [45], CYP2A6 [79], and CYP158A1 [80]. The presence of substrates can significantly impede the access of O_2 and other diatomic ligands to the heme iron, and the scale of this effect can vary to a great extent with various substrates. For instance, different oxygen binding rates have been reported for CYP158A1 saturated with flaviolin (120 s^{-1}) or 2-hydroxy-1,4-naphthoquinone (15 s^{-1}) [80]. The effect of substrates on oxygen binding and autoxidation have been systematically studied for monomeric CYP3A4 incorporated into 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) Nanodiscs [81, 82]. The experimentally observed rate of O_2 binding in the presence of testosterone TST and bromocryptine (BC) varies by more than an order of magnitude (350–400 s^{-1} with TST and 24 s^{-1} with BC at 279 K). The effect of TST is even more dramatic with respect to the binding of cyanide to CYP3A4, which is 60 times slower in the presence of substrate than in its absence [82], as is also observed for the association of small ligands like cyanide and imidazole to the ferric enzyme [83, 84] and of carbon monoxide to ferrous P450. [85]. The rate of CO binding to CYP101A1 in the absence of camphor (5×10^6 M^{-1} s^{-1}) is two orders of magnitude faster than in its presence ($4\cdot10^4$ M^{-1} s^{-1}) [86]. A similar slowing of CO binding by substrate was observed for CYP108, but not for CYP102 [87]. This reduction in the

binding rates of diatomic ligands in the presence of substrates is commonly observed with other heme enzymes, including indoleamine 2,3-dioxygenase (IDO) [88] and nitric oxide synthase (NOS) [89].

An interesting aspect of the kinetics of CO binding to CYP102A1 has been described by Munro et al. [90] using laser photooxidation of NAD(P)H to reduce the heme iron in the microsecond timescale ($k_{obs} = 14{,}000\ s^{-1}$), a much faster rate than the typical dead time in stopped-flow studies (~1 ms). The surprisingly fast CO binding observed in this work, with apparent rates of 1700–3000 s^{-1}, was attributed to the presence of CO molecules inside the protein in the immediate vicinity of the heme iron. In this case, there is no need for penetration of the diatomic ligand from the solution into the substrate-binding pocket and diffusion towards the heme iron. It is reasonable to expect that the same may be true for other diatomic neutral gases, such as O_2, and as a result the oxygen-binding step may happen in aerobic solution with apparent rates significantly higher than those measured in stopped-flow experiments, where the reduced protein is equilibrated with deoxygenated buffer before mixing with oxygenated solvent and oxygen must access the heme from outside. The same effects have also been described for CYP121 and CYP51B1 from *Mycobacterium tuberculosis* [91].

The first X-ray structure of the ferrous dioxygen (or ferrous-oxy complex) of a cytochrome P450 was solved by Schlichting and coworkers in 2000 using the bacterial CYP101A1 [92]. The oxygen molecule was found to fit tightly between the substrate camphor and the small cleft in the I-helix as suggested by the structure of the ferric protein [33]. Importantly, the binding of dioxygen resulted in a change in the active site hydrogen-bonding structure through the addition of two new water molecules not observed in the ferric structures. These are illustrated in Fig. 3.4. The appearance of these water molecules in the oxygenated form of CYP101A1 strongly suggested a likely path for the delivery of protons to the distal oxygen atom of the heme bound O_2. This provided the first structure-based suggestion of a mechanism of oxygen activation in the cytochromes P450 [74] through site-specific proton delivery to the distal atom of the dioxygen ligand [93–95]. A first proton transfer would lead to the hydroperoxide intermediate and a second proton delivery would then lead to cleavage of the O–O bond, releasing a molecule of water and generating the higher valent compound I oxidizing species. Oxy-complex structures of wild type and

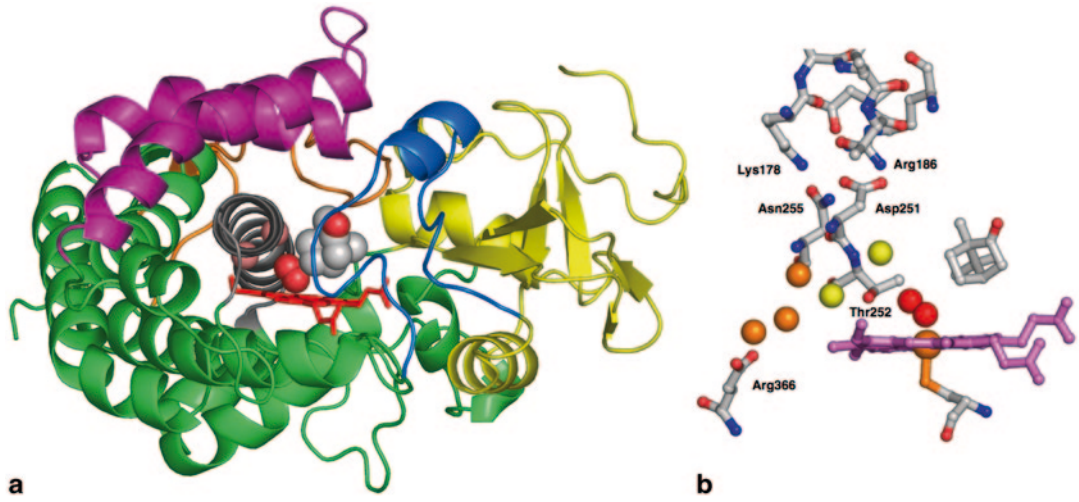

Fig. 3.4 **a** X-ray structure of the oxy-complex of CYP101A1 (1DZ8.pdb [92]) with two new water molecules appearing in the cleft opening in the I-helix next to the coordinated dioxygen molecule. **b** A tentative proton delivery pathway with two new water molecules is shown in *yellow*

mutant CYP101A1 [74] and P450eryF [73] were subsequently solved by Poulos and coworkers.

Because the resolution of the structures of oxygenated cytochromes P450 is not high enough for precise evaluation of the geometric parameters of the heme ligands, information about bond lengths and angles can be best obtained from the structures of closely related model complexes. A comprehensive review published in 1994 [96] provides an extensive description of the physical inorganic chemistry of heme oxygen complexes. For many years, the classical reference for the geometric parameters of the iron–porphyrin oxy-complex was the X-ray crystallographic study by the Collman group [97] of two picket-fence iron porphyrins with imidazole and dioxygen as axial ligands. These structures provided a clear picture of the end-on coordinated dioxygen molecule with a Fe–O–O angle of 135°–137° and O–O bond lengths of 1.23 and 1.26 Å. These structures also revealed significant mobility and multiple orientations of the coordinated dioxygen, both in plane and out of plane together with the axial histidine ligand [97, 98]. Recently, a new high-resolution structure of the oxy-complex of an iron picket fence porphyrin has been determined and the oxidation state of the iron atom was characterized by temperature dependent Mössbauer spectroscopy [99] and provided the geometric parameters of a heme iron end-on coordinated dioxygen with the highest precision. The values determined are Fe–O = 1.811 Å, Fe–O–O = 118.2°, and O–O = 1.281 Å, and an off-axis tilt of 6.2° in the complex with 2-methyl imidazole as the axial ligand. This O–O distance is in good agreement with the range expected from the Fourier transform infrared spectroscopy (FTIR) experimental frequencies of the O–O stretch mode observed for such model complexes (1150–1163 cm^{-1}) [100] and with the general dioxygen—superoxide—peroxide formal assignment [96, 101]. Similar bond lengths for Fe–O (1.81–1.83) Å and O–O (1.24–1.25) Å are reported in two high-resolution X-ray structures of the oxy-complex of sperm whale myoglobin [102, 103]. For reference, in various models the O–O bond length increases from 1.21 Å in dioxygen to 1.33 Å in the superoxide anion [104], and to 1.49 Å in the peroxide anion [96], concomitant with reduction of the O–O bond order from 2 to 1.5 to 1.

Another recent and important study of the oxy-complex of the picket-fence iron-porphyrin model combined the L edge extended X-ray absorption fine structure (EXAFS) and density functional theory calculations with a goal of characterizing the electronic structure of iron in this complex [105]. Comparison of X-ray absorption spectra (XAS) results obtained for the oxy-complex of several other hexa-coordinated ferrous and ferric low-spin complexes revealed strong σ-donation and strong π-interaction of the dioxygen moiety with iron, indicating a highly covalent Fe–O bond. This fact restricts the formal application of the oxidation state formalism and explains the absence of the hole in the dπ orbital of the iron, which is characteristic of all low-spin ferric complexes. XAS spectra of the oxy-complex are similar to the spectra of the *bis*-imidazole ferrous porphyrin, (Fig. 12 in Ref. [105]) and do not look like the spectra of ferric complexes [105]. However, the electronic configuration in the oxy-complexes strongly depends on the presence or absence of hydrogen bonds to the coordinated oxygen [103]. In the model porphyrin complexes there is no hydrogen bonding [105], while in most heme proteins there are proton-donating amino acid side chains or water molecules that can form one or two hydrogen bonds and shift the electron density towards the ferric-superoxide configuration [103].

Local interactions in the immediate vicinity of the heme and axial ligands can strongly affect the electronic structure of the oxy-complex. These can be detected by comparison of the ultraviolet–visible (UV–vis) spectra of various oxygenated cytochromes P450, nitric oxide synthase (NOS), and chloroperoxidase (CPO), which all have identical iron coordination spheres and the same heme prosthetic group. While all display a split Soret band [106, 107], the position of the main band changes from 418 nm in CYP101A1 [77] to 430 nm in CPO [108]. Even for the same cytochrome P450 the position of the main Soret band may vary significantly in the presence of various substrates, as documented for CYP102A1 (422–425 nm) [36, 109] and for CYP3A4 (420–

425 nm) [81, 110]. In CYP2B4 the UV–vis and magnetic circular dichroism (MCD) spectra of the oxy-complex with and without substrate are very similar, with the Soret maximum at 423 nm [111]. However, a red shift of the Soret band to 426–427 nm is observed in the CYP2B4 E301Q and T302A mutants, respectively, indicating slightly different configurations of the hydrogen-bonding network caused by these mutations. This is in contrast to the same mutations (D251N and T252A) in CYP101A1, where no changes in the UV–vis and MCD spectra were observed relative to the wild-type protein [112].

The most detailed and site-specific information on the bond strength and hydrogen-bonding environment of the coordinated dioxygen, as well as on the main heme vibrational modes, can be obtained using resonance Raman (rR) spectroscopy [113]. Because of the limited stability of the oxy-complex at ambient conditions, most Raman measurements are performed under cryogenic conditions using frozen solutions. The first successful rR characterization of the oxy-complex of CYP101A1 in the presence of camphor was published in 1986 [71]. A strong O–O mode at 1140 cm^{-1} was identified based on isotopic shift of this band using $^{16}O_2$ and $^{18}O_2$ of 1121–1131 cm^{-1}, near that reported for isolated superoxide ions in solid matrices [104]. Note that the O–O stretching mode is usually not active in rR spectra of heme proteins that have histidine as a proximal iron ligand, although it was identified in infrared (IR) spectra of the oxy-complexes of hemoglobin and myoglobin at 1135 cm^{-1} [114]. However, in some cases the O–O stretch mode was experimentally observed, i.e., in oxyhemoglobins from *Chlamydomonas* (1136 cm^{-1}) and from *Sinechocystis* (1133 cm^{-1}) [115], and also in indoleamine dioxygenase (IDO) (1138 cm^{-1}) [116]. Subsequent rR spectra of oxy-complexes in CYP101A1 provided new information on perturbation of the Fe–OO moiety by various substrates [72] and by Pdx [117].

The rR spectra of the oxy-complexes of several human cytochromes P450 have also been measured recently for recombinant purified CYP11A1 [118] and for purified CYP17A1 [119] and CYP19A1 incorporated in Nanodisc bilayers [120]. In general, all features of these spectra are similar to those previously reported for CYP101A1. The position of the O–O mode varies from 1147 to 1124 cm^{-1} and the range of the Fe–OO mode frequencies is between 540 and 529 cm^{-1}, with the expected linear correlation observed [118, 121–123]. The positions of these modes are not significantly different in the thiolate-ligated cytochromes P450 and nitric oxide synthases, but can be substantially perturbed by hydrogen bonding to the dioxygen ligand [120, 124–126] and by steric effects caused by size and positioning of substrates [72]. In addition, detailed analysis of the spectra of oxy-complexes in the presence of various substrates revealed a striking difference in the configuration of the hydrogen-bonding network that includes the hydroxyl group of the substrate, the coordinated dioxygen moiety, and possibly other amino acid side chains and active site waters. For example, based on the different pattern of perturbations of the O–O and Fe–OO modes by 17-hydroxypregnenolone and 17-hydroxyprogesterone, hydrogen bonding to the proximal oxygen atom for the former and the distal oxygen atom for the latter, has been observed in CYP17A1 [119]. This difference correlates with the efficiency of the lyase reaction catalyzed by CYP17A1 and speaks directly to the intermediate states involved in the catalytic cycle and the identity of the "active oxygen" involved. More information about the Fe–O vibrational modes, as well as detection of new modes not seen in rR spectra, was provided by nuclear resonance vibrational spectroscopy (NRVS) [127, 128]. Using this method, Sage and collaborators demonstrated the strongly mixed character of two Fe–O modes observed in Raman spectra and claimed that the unambiguous assignment of these modes to either bending or stretching vibrations is not always valid.

The ferrous dioxygen complexes of heme proteins are not stable species, with the overall lifetime of this state in the cytochromes P450 ranging from milliseconds to minutes (Table 3.1). Autoxidation of the $Fe–O_2$ complex proceeds through spontaneous dissociation of superoxide, which in turn quickly dismutates into hydrogen peroxide and dioxygen in aqueous solution. The heme is returned to the resting ferric state. The rates of

Table 3.1 Experimentally observed autoxidation rates for cytochromes P450

P450	Substrate	Conditions (K)	Rate (s^{-1})	Source
CYP101A1	−sub	275–299	0.002–0.03	[136]
	+cam	278–293	0.0003–0.0043	[87]
T252A, V	+cam	293	0.005–0.01	[313]
G248A	+cam	283	0.003–0.005	[272]
	+cam	298	0.004	[189]
CYP102A1	+sub	277–293	0.025–0.22	[87]
	−sub	288	0.09	[37]
	+sub	288	0.06	[37]
F393H	−sub	288	0.018	[37]
	+sub	288	0.0013	[37]
T268A	−sub	288	0.27	[37]
	+sub	288	0.26	[37]
CYP108	+sub	277–293	0.0007–0.017	[87]
CYP119	−sub	278	0.08	[314]
CYP158	+sub	296	0.042–0.09	[80]
P450a, b,c[a]	−sub	277	1.6–5	[315]
CYP1A2	−sub	277	0.41	[304]
E318D	−sub		0.80	[304]
E318A	−sub		0.07	[304]
T319A	−sub		0.37	[304]
CYP2A6	+sub	296	0.3	[79]
CYP2B4	+sub	288	0.09	[316]
CYP3A4	−sub	278–302	20–140	[81]
	Testosterone	279–310	0.37–20	[81]
	Bromocriptine	279–310	0.12–2.5	[81]
CYP11A1	+cholesterol	275	0.0063	[130]
	+dihydrocholesterol		0.0004	[130]
CYP19A1	+androstenedione	298–310	0.21–0.7	[317]
CPO	−subs	298	1.7	[318]
iNOS, H_2B	−subs	283	0.3	[319]
W188H, H_2B	−subs	283	0.0044	[319]
nNOS, -pterin	−subs	283	0.14	[320]

cam 1*R*-camphor, *CPO* chloroperoxidase, *H_2B* dihydro-biopterin, *iNOS* inducible nitric oxide synthase, *nNOS* neuronal nitric oxide synthase

[a] Fractions of cytochromes P450 purified from *Rhizobium japonicum*

autoxidation strongly depend on the presence of substrate, which sometimes can extend the half-life of the oxy-complex by a factor of 100 [81]. Another common property of the oxy-complexes in cytochromes P450 is a strong temperature dependence of autoxidation, with high activation energies implying substantial conformational changes involved in the release of superoxide [50, 129–132]. For this reason the oxy-complexes of substrate-free cytochromes P450 are prepared at low temperatures, often with the help of cryosolvents to suppress the freezing point and extend the temperature range for solutions down to 250–240 K [109, 111, 133–138]. The observed stabilization of the oxy-complexes in the presence of substrate is a general property of cytochromes P450 and is usually attributed to steric restrictions for superoxide escape from the active site. The concept of conformational gating is also supported by a similar slowing of the dissociation rates of CO, CN^- and other diatomic ligands in the presence of a substrate. The same mechanism can be observed even when the substrate is present far from the catalytic site, as evidenced

in human CYP3A4 when steroids bind at a peripheral allosteric site [82]. For CYP3A4, which can bind up to three TST molecules, the substrate dependence of the autoxidation rate is not trivial, with the major stabilization of the oxy-complex caused by the first binding event. Although the first TST molecule is likely bound at the same peripheral binding site as progesterone in the crystal structure described by Williams et al. [139], with no spin shift and no product formed at this stage [140], both autoxidation and geminate rebinding of CO undergo substantial changes and almost reach saturation with no changes caused by the second and third substrate binding [82]. Together with the high activation energies observed for autoxidation (15–18 kcal/mol in CYP3A4 with and without substrates) [81], these results suggest the existence of "conformational gating" in the binding and dissociation of diatomic ligands in various cytochromes P450. The presence of open and closed forms in equilibrium is now considered as a common property of the cytochrome P450 fold [95, 141] (see also Chap. 1 by Johnson and Poulos) and substrate binding is known to strongly affect the position of this equilibrium [142–145] as well as the likely rates of transitions between these states. Apparently, substrate binding to the peripheral binding site in CYP3A4 can play an effector role by stabilizing the closed form and thereby significantly decreasing the dissociation rate of diatomic ligands, as well as possibly other substrate or product molecules, from the active site. Manifestations of such effects of substrate or effector binding at the peripheral sites were observed as substrate or product inhibition at high substrate concentrations in other P450s such as CYP3A4 [146] and CYP2E1 [147].

Autoxidation together with direct peroxide dissociation from cytochromes P450 is responsible for the formation of reactive oxygen species and their formation is suggested to be an important source of toxic and potentially carcinogenic compounds [148, 149]. In some cases autoxidation is the main uncoupling pathway, as in CYP3A4 with poorly coupled substrates, for which autoxidation is faster than the second electron transfer. This is suggested based on the very fast autoxidation rates, for example 20 s^{-1} with TST bound at 37 °C, as compared with the relatively slow overall steady-state NADPH consumption rate (about 4 s^{-1} under the same conditions) [81, 82]. Substrate binding significantly stabilizes the oxy-complex by both kinetic and thermodynamic mechanisms. Kinetic stabilization due to steric restriction of the escape pathway for superoxide in the presence of substrate was mentioned in the previous section. The thermodynamic stabilization is due to the changes in redox potential of the heme iron as described [36, 132]. The oxy-complex can decompose via dissociation of dioxygen from the ferrous heme, or by dissociation of superoxide anion from the ferric heme, as shown in Fig. 3.5. The overall process can be represented by two steps, fast equilibration in the immediate vicinity of the heme inside the active site, and slower escape of diatomic ligand into the solvent.

Fig. 3.5 Decomposition pathways of the Fe–O_2 complex *via* reversible dissociation of dioxygen from the ferrous heme (*top*) or quasi-irreversible dissociation of superoxide from the ferric heme

Here the first reversible steps, breakage of the coordination bond, and geminate rebinding of the neutral dioxygen (top pathway) or superoxide (bottom pathway), are equilibrated on the ~10 ns timescale [150, 151]. The relative probability of superoxide dissociation is determined by the partitioning constant K_{part}, which can be calculated by combining the two redox equilibria in Fig. 3.6:

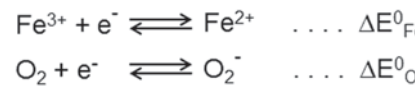

Fig. 3.6 Redox equilibria between ferrous and ferric states in the heme iron and between dioxygen and superoxide

The fraction of dioxygen dissociating from the protein via the bottom pathway as superoxide can be calculated as shown in the equation below:

$$K_{\text{part}} = \frac{K_1}{K_2} = \frac{[\text{Fe}^{3+}\ldots O_2^-]}{[\text{Fe}^{2+}\ldots \text{O}_2]} = \exp\left(\frac{\Delta E^0_{\text{O}_2}}{\text{RT}}\right)\exp\left(\frac{-\Delta E^0_{\text{Fe}}}{\text{RT}}\right)$$

Here the midpoint potential of the dioxygen–superoxide pair is −0.33 V for unprotonated superoxide [101], so the first term is constant, and partitioning between the autoxidation pathway and reversible dissociation of dioxygen depends exponentially on the midpoint redox potential of the heme iron. As a result, the observed apparent autoxidation rate k_{autox} also increases exponentially when the redox potential of the heme iron decreases:

$$K_{\text{autox}} = \text{d}[\text{Fe}-\text{O}_2]/\text{d}t = [\text{Fe}-\text{O}_2]\,K_{\text{p}}k_{\text{o}} \sim \exp\left[\left(\Delta E^0_{\text{O}_2} - \Delta E^0_{\text{Fe}}\right)/\text{RT}\right]$$

This exponential dependence of the apparent autoxidation rates on the midpoint redox potential of the heme iron in cytochromes P450 explains the higher stability of the oxy-ferrous intermediates in the presence of substrates [36, 37, 132]. In addition, the presence of substrate at the active site of the cytochrome P450 creates steric restrictions on the mobility of diatomic ligands and furthermore increases the lifetime of oxy-complexes and thus improves the efficiency of the overall catalytic cycle by reducing unproductive dissociation of superoxide. [81, 82]. Overall, this regulatory role of substrate on the efficiency of oxygen activation is a critical factor in the mechanism of cytochrome P450.

3.6 Second Electron Transfer and the Peroxo- and Hydroperoxo-Intermediates

The rate of the second electron transfer, **[4] → [5]** in Fig. 3.1 is difficult to measure. The marginal stability of the oxy-complex in many cases is a serious obstacle, made more difficult by the rate-limiting formation of a productive complex with the protein redox partner, either an iron–sulfur ferredoxin (e.g., Pdx), or the flavoprotein cytochrome P450 reductase. In several experimental studies the reduction rates of oxy-complexes in cytochromes P450 were measured using stopped-flow absorption spectroscopy by monitoring the decay of the oxy-complex after rapid mixing with the reduced redox partner [152–156]. The measured rates varied greatly, from $>100\ \text{s}^{-1}$ for the fast CYP101A1 reduction by Pdx, to 8.4 and $0.37\ \text{s}^{-1}$ for the slower and multiphasic reduction of CYP2B4 by cytochrome P450 reductase (CPR). Steady-state kinetic studies, conducted over many years, did not reveal any detectable spectral intermediate following the second electron transfer to the oxy-complex before the appearance of the ferric resting state. From the first investigations using the soluble CYP101A1, it was apparent that this second electron transfer is at least partially rate limiting, as the ferrous-oxy complex accumulates to some degree during turnover. The same observations were made by monitoring the steady-state turnover of microsomal cytochromes P450 [157] and in stopped-flow spectroscopic studies of oxygen binding to purified microsomal cytochromes P450 [158]. Thus, despite numerous attempts, no success has been achieved in cleanly observing a peroxo- or hydroperoxo-ferric intermediate in wild-type P450 at room temperature with the normal redox partners and atmospheric dioxygen. Early stopped-flow studies did claim the observation of such an intermediate state [159, 160], and with the D251N mutant of CYP101A1, where protonation is impaired, some level of a peroxo- or hydroperoxo-ferric species could be observed [161]. As will be discussed, the spectral and structural characterization of these intermediates in the cytochromes P450, as well as in other oxygen reactive heme proteins, requires the use of cryoradiolytic reduction. A shunt pathway exists, however, wherein two oxygen atoms and two reducing equivalents can be brought to the ferric heme together in the form of a peroxide or peroxy acid. With this approach, a transient species

with red-shifted Soret band has been observed in horseradish peroxidase [162, 163].

Since the early 1970s, it was understood that one-electron reduction of the ferrous-oxy complex would generate a state with two redox equivalents and dioxygen—a ferric–"peroxo" state, with the electron going somewhere in the liganded prosthetic group. As the oxygenated intermediate has a dominant "ferric-superoxo" resonance form, as evidenced by Mossbauer measurements [75], addition of the second electron was thought to form a ferric iron with the oxygen reduced to the level of peroxide in resonance with a ferrous-superoxo configuration. However, neither the peroxo- **[5a]** nor hydroperoxo-ferric **[5b]** complexes has ever been cleanly observed at ambient temperatures. This intermediate, termed "compound 0" by analogy to similar states in the peroxidases, undergoes further transformation and disappears faster than it is formed. A pioneering breakthrough was realized though the work of Davydov, wherein the oxygen intermediate was trapped in a frozen matrix and the second electron was added by radiolysis. Although low-temperature matrix-isolation techniques were well established in the 1950s and 1960s [164–168], the first applications of this method to heme protein solutions were those of Davydov [169–174] and Symons [175–179]. Cryoradiolysis uses ionizing radiation to produce hydrated electrons, which can either interact directly with the protein molecule or the solvent matrix. For dilute protein solutions, the volume fraction of the mixed solvent is much larger and hence the predominant effect is to produce hydrated electrons that are highly mobile even at cryogenic temperatures. The radiation chemistry of aqueous solutions has been well studied, with reviews focused on frozen aqueous solutions of proteins also appearing in the literature [134, 174, 180, 181]. It is worth noting that the solvent itself also plays a crucial role as a selective quencher of undesired radiolysis products. For example, glycerol or ethylene glycol efficiently trap and quench hydroxyl radicals in the cryogenic radiolytic reduction of metalloproteins, with the result that a higher net yield of solvated electrons is available to reduce the proteins of interest [182].

A pioneering publication in the P450 literature was that of Davydov, Huttermann, and Peterson, who demonstrated that radiolytic reduction of the ferrous dioxygen complex of CYP101A1 at liquid nitrogen temperature yielded an electron paramagnetic resonance (EPR) signal identified as a peroxo intermediate [183]. Although referenced in several reviews of the P450 mechanism, it was not until Davydov moved to the Hoffman laboratory that electron nuclear double resonance (ENDOR), and additional magnetic resonance investigations at variable frequencies, spectroscopically defined the peroxoanion and hydroperoxo forms of ligated heme. Since that time cryoradiolytic reduction of oxy-complexes has been the method of choice for stabilization of the fleeting intermediates in the P450 catalytic cycle with the goal of obtaining the detailed structural and spectroscopic information necessary for evaluation of the mechanism of oxygen activation and metalloenzyme catalysis. Several reviews on experimental applications of these methods and the results obtained using the cryoradiolytic approach have been published recently [134, 135, 180, 184]. For CYP101A1, radiolysis at 77 K trapped the hydroperoxo intermediate [185]. In the D251N mutant of CYP101A1, which altered the occupancy of active site waters as observed in the crystal structure of the ferrous dioxygen complex [92, 186], the species observed upon 77 K radiolysis was the peroxoanion. Thermal annealing of this trapped state, with monitoring by EPR and ENDOR spectroscopy [185], allowed direct observation of the protonation event and quantitative conversion of the peroxoanion to the hydroperoxo and product.

These first detailed characterizations of the peroxo- and hydroperoxo-ferric intermediates in CYP101A1 [185, 187] provided several important results and enabled further experimental studies with other heme proteins. Clear EPR signatures for the unprotonated peroxo-ferric ($g_1 < 2.27$) and protonated hydroperoxo-ferric ($g_1 > 2.27$) intermediates in cytochromes P450

were thus defined. These parameters are very similar to those observed in other heme proteins, such as myoglobin, hemoglobin, and horseradish peroxidase (see Table 3.2). Thus, the first immediate product of cryoradiolytic reduction of the oxy-complex is the peroxo anion, as proton transfer events are prevented at low temperature. In some cases, such as with wild-type CYP10A1, protonation can occur even at 77 K and one needs to do the cryoreduction at helium temperatures to trap the peroxo anion. This temperature dependence of the proton transfer events provides a recipe for stepwise annealing of the trapped peroxo anion to follow the transformation of metastable intermediates along the reaction coordinate through to product formation. The catalytic competence of the cryoradiolytic reduction of CYP101A1 can be directly demonstrated by analysis of product formation, with the overall yield proportional to the irradiation dose [185].

Subsequent work with CYP101A1 demonstrated that various substrates significantly modu-

Table 3.2 EPR parameters of (hydro)peroxo-ferric complexes in heme proteins

Heme protein	*g* values	Assignment	Reference
Myoglobin	2.218, 2.118, 1.966	Fe^{3+}–OO^{2-}	[321]
	2.21, 2.11, 1.97	Fe^{3+}–OO^{2-}	[322]
	2.30, 2.16, 1.94	Fe^{3+}–OOH^{-}	[322]
Hemoglobin			
α-subunit	2.213, 2.121, 1.968	Fe^{3+}–OO^{2-}	[321]
β-subunit	2.22, 2.13, 1.97	Fe^{3+}–OO^{2-}	[321]
	2.25, 2.15, 1.966	Fe^{3+}–OO^{2-}	[321]
β-chain	2.31, 2.19, 1.948	Fe^{3+}–OOH^{-}	[321]
Indoleamine dioxygenase	2.32, 2.17, 1.947	Fe^{3+}–OOH^{-}	[323]
Indoleamine dioxygenase +subs	2.27, 2.17, 1.946	Fe^{3+}–OO^{2-}	[323]
Tryptophan dioxygenase +subs	2.27, 2.17, 1.95	Fe^{3+}–OO^{2-}	[323]
Peroxidase	2.08, ?	Fe^{2+}–OO^{-}	[322]
	2.31, 2.16, 1.95	Fe^{3+}–OOH^{-}	[322]
	2.27, 2.18, 1.90	Fe^{3+}–OO^{2-}	[200]
	2.32, 2.18, 1.90	Fe^{3+}–OOH^{-}	[200]
Dehaloperoxidase	2.25, 2.15, 1.963	Fe^{3+}–OO^{2-}	[324]
	2.32, 2.18, 1.945	Fe^{3+}–OOH^{-}	[324]
Heme oxygenase	2.37, 2.19, 1.93	Fe^{3+}–OOH^{-}	[325]
Nitric oxide synthase			
eNOS +Arg	2.26, 2.16, nd	Fe^{3+}–OO^{2-}	[191]
gsNOS +Arg	2.27, 2.18, nd	Fe^{3+}–OO^{2-}	[326]
	2.31, 2.16, nd	Fe^{3+}–OOH^{-}	[326]
Cytochrome P450			
CYP101A1 −subs	2.355, 2.212, 1.935	Fe^{3+}–OOH^{-}	[188]
CYP101A1 +cam	2.30, 2.16, 1.96	Fe^{3+}–OOH^{-}	[185, 187]
CYP101A1, T252A +cam	2.306, 2.173, 1.956	Fe^{3+}–OOH^{-}	[188]
CYP101A1, D251N +cam	2.25, 2.16, 1.96	Fe^{3+}–OO^{2-}	[185, 187]
CYP101A1 +adamantanone	2.257,2.16, nd	Fe^{3+}–OO^{2-}	[188]
	2.30, 2.162, ~1.96	Fe^{3+}–OOH^{-}	[188]
CYP101A1 + 5-methylenyl camphor	2.296, 2.157, 1.957	Fe^{3+}–OOH^{-}	[188]
CYP101A1, G248T, G248V +cam	2.24, 2.16, 1.94	Fe^{3+}–OOH^{-}	[189]
CYP2B4 +BHT	2.32, 2.18, 1.94	Fe^{3+}–OO^{2-}	[133]
CYP11A1 +cholesterol	2.214, 2.14, nd 2.34, 2.182, 1.949	Fe^{3+}–OOH^{-}	[195]
CYP19A1 +AD	2.254, 2.163, nd	Fe^{3+}–OO^{2-}	[194]

late proton delivery to the coordinated dioxygen, as monitored by EPR and ENDOR of the cryoreduced oxy-complexes in the presence of different substrates [188]. Controlled annealing at elevated temperatures (170–180 K) demonstrated that the presence of any substrate dramatically increases the stability of the hydroperoxo-ferric complex, with lifetimes at least 20 times longer than in the absence of a substrate. With all substrates, alternate and multiple conformational substates have been detected in the heme-iron center by changes in $^{14}N,^{1}H$ hyperfine couplings in the ENDOR spectra. Unusual EPR and ENDOR spectra and reactivity have been observed with CYP101A1 bound with (1*R*)-methylenyl camphor. One well-defined conformational substate of the heme was observed, but the decay rates of the hydroperoxo-ferric complexes of wild-type CYP101A1 and its T252A mutant at 180 K were much lower than with all other substrates. Although the T252A mutant does not yield a product with normal substrates, in this case the epoxide of (1*R*)-methylenyl camphor was generated. Dawson, Hoffman, and colleagues thus suggested that there may be a direct involvement of compound 0 in the epoxidation reaction, rather than a reaction involving proton transfer, O–O bond scission and compound I formation. The results of this work suggest a potential for the involvement of substrates in modulating the chemical properties of peroxo- and hydroperoxo-ferric intermediates and selection of the appropriate "active oxygen" for catalysis. Such a role may help explain the numerous proposals for the involvement of multiple oxidants and catalytic mechanisms in P450 function [56].

In addition to the critical acid–alcohol pair that is directly involved in the protonation of peroxo-ferric complexes in cytochromes P450, other amino acids in the immediate vicinity also can perturb the proton delivery and significantly change the functional properties of the enzyme. The CYP101A1 single G248 mutants [189], which retain the native acid–alcohol pair of D251 and T252, show significant perturbation of the proton delivery, although both mutant proteins still catalyze camphor hydroxylation in a reconstituted system. Functional studies suggest that the second protonation of the hydroperoxo-anion is inhibited by mutations at the 248 position. EPR of the cryoreduced oxy-complex shows that the first protonation is also impeded, since the immediate product of the cryoreduction at 77 K is almost completely the unprotonated peroxo-anion, in contrast to the wild-type and the T252A mutant, for which cryoreduction at 77 K produces the hydroperoxo state [185, 187, 190].

With the low-temperature oxygenation protocols developed for the preparation of unstable oxy-complexes in cytochromes P450 and NOS [108, 109, 135, 180, 191–193], cryoradiolytic reduction and characterization of the peroxo- and hydroperoxo-ferric intermediates have been realized for the mammalian CYP2B4 [133] and the steroid metabolizing P450s CYP17A1, CYP19A1 [194], and CYP11A1 [195]. In addition to the substrate free protein, samples of CYP2B4 have been prepared in the presence of two substrates, benzphetamine (BP) and 3-hydroxy-*tert*-butyl toluene (BHT). Because no high-spin signal was detected by EPR in the frozen solution of CYP2B4 with BP, dissociation of the substrate at low temperature in the cryosolvent (60 % glycerol with Tris buffer, pH 8.0) was suggested, in contrast to CYP2B4 bound with BHT, which revealed a mostly high-spin EPR signal. Oxygenation of the reduced protein was realized at −40 °C in order to minimize autoxidation [109, 111]. The yield of hydroperoxo-ferric complex was been estimated at ~40 % by comparison of the EPR signal with the calibrated standard [133]. As with CYP101A1 and heme oxygenase [196], the immediate product of cryoradiolytic reduction in CYP2B4 with or without substrate was the already protonated hydroperoxo-ferric complex characterized by $g_1 > 2.27$.

The peroxo- and hydroperoxo-ferric intermediates in the mammalian cholesterol side-chain cleaving cytochrome P450 (CYP11A1) has been recently documented [195]. The oxy-complex of CYP11A1 with cholesterol bound was radiolytically reduced at 77 K in 33 % glycerol/phosphate buffer at pH 7.5. After irradiation the main cryoreduced intermediate had an EPR signal with $g_1 = 2.34$ characteristic of a protonated hydroperoxo-ferric complex. However, two minor

signals with $g_1 = 2.214$ and $g_1 = 2.28$ indicated the presence of some unprotonated peroxo-ferric intermediates. These latter intermediates both converted to the hydroperoxo-ferric intermediate after annealing at 145 K, with a considerable protium/deuterium (H/D) solvent isotope effect for the conversion of the 2.214 signal, but no isotope effect for the reaction of the 2.28 intermediate. Taken together, these observations indicate the presence of multiple conformers of coordinated dioxygen and one or more water molecules in the immediate vicinity that may serve as proton donors to the peroxo-anion in CYP11A1. Annealing at 185 K and further to 220 K, resulted in decay of the hydroperoxo-ferric intermediate and formation of the 22*R*-hydroxycholesterol product. This step also featured a substantial solvent H/D isotope effect consistent with the expected partially rate-limiting second proton-transfer step, which is necessary for formation of the catalytically active compound I. This suggests that the C–C bond scission of a vicinal diol, as is the case in the generation of pregnenolone from cholesterol by CYP11A1, uses compound I as the "active oxygen" for catalysis.

Generation and decay of peroxo-states can also be monitored by optical absorption spectroscopy [137, 180, 197, 198], although with UV–vis methods it is not possible to differentiate between the peroxo and hydroperoxo intermediates [198]. The main spectral feature of these intermediates in cytochromes P450 and in other thiolate-ligated proteins is a significant red-shift of the Soret band from 420–430 to 440–450 nm, and the appearance of a second minor band at ~375 nm. These properties are consistent with the split Soret band characteristic of the optical spectra of the ferrous O_2 and CO complexes and the ferric-cyanide adduct of cytochrome P450 [106, 107, 199]. Interestingly, only a minor red-shift of the Soret band (3–8 nm) is observed for the peroxo-complexes in heme proteins with histidine as the proximal iron ligand [134, 192, 200, 201].

As noted in the previous discussion of the earlier intermediates in the P450 reaction cycle, rR spectroscopy is a powerful tool to reveal critical information regarding P450 structure and function, including mechanistic details of P450 catalytic oxygen activation and substrate metabolism and their linkage to the delivery of protons to the reduced heme-dioxygen complex. rR spectroscopy probes the vibrational modes associated with the active site, is operational in all states of the reaction wheel, and hence is uniquely positioned to provide key information on the mechanism of "oxygen activation" in cytochromes P450.

Low-temperature rR investigations have been extensively conducted by the Kincaid laboratory on radiolytically reduced oxy-ferrous cytochromes P450 [183, 185, 187, 188, 197, 198, 201–205]. Using the D251N mutant of CYP101A1 and experiments analogous to the EPR investigations already discussed, the peroxoanion intermediate and the formation of the protonated hydroperoxo state following thermal annealing was characterized. These studies demonstrated that the hydroperoxo-anion retains the end-on structure of the oxy-ferrous precursor and forms a relatively strong bond with the heme iron that is characterized by ν(Fe–O) ~617 cm^{-1} in the hydroperoxo-ferric complex in myoglobin [201] and 564 cm^{-1} in CYP101A1 [198, 205], while the unprotonated ferric-peroxo complex **[5a]** in the D251N mutant of CYP101A1 displays a slightly weaker Fe–O bond with ν(Fe–O) 553 cm^{-1} [198]. These complexes reflected the typical features of low-spin heme-thiolate complexes with a narrow span of *g* values in the EPR spectra and a red-shifted split Soret band with maxima at 436–440 and 370–375 nm [185, 197, 204].

Spectroscopic and theoretical studies reveal that the length and strength of the O–O bond in the peroxo states (termed Compound 0 by analogy to the peroxidase literature) are similar to those observed in the low-spin oxygen activating nonheme (hydro)peroxo-ferric complexes. Particularly interesting is the direct observation of the downshift of ν(O–O) from 792 cm^{-1} in the peroxo anion state (**[5a]** in Fig. 3.1) to 774 cm^{-1} in the hydroperoxo **[5b]**, indicating a weakening of the O–O bond as a result of protonation of the peroxo-anion coordinated to iron [198]. Notably, the ν(O–O) in the P450 peroxo-ferric intermediates is significantly lower than in myoglobin with cobalt-substituted heme, where this mode was

observed at 851 cm^{-1} [206]. This difference is attributed to the strong electron donating capabilities of the thiolate proximal ligand in cytochrome P450 as compared to the imidazole nitrogen of the proximal histidine in myoglobin. The thiolate *trans*-effect weakens the O–O bond and promotes its heterolytic cleavage, with concomitant formation of the high-valent catalytically active ferryl-oxo intermediate (**[6]** in Fig. 3.1). However, the presence of the distinct hydroperoxo-ferric heme intermediate in the frozen solutions and in crystals of cytochromes P450 and other heme proteins suggests that there is no spontaneous breakage of the O–O bond, but rather the enzyme/substrate provides a catalytically important function. Thus, efficient formation of the main active intermediate Compound I requires catalytic delivery of the second proton to the distal oxygen atom (Fig. 3.1 **[5b] → [6]).** The application of cryoreduction and annealing of native and mutant proteins, with concerted spectroscopic characterization by EPR/ENDOR and Raman spectroscopy, offers a means for revealing these critical steps in oxygen activation by the cytochromes P450.

Additional information on the structure and reactivity of peroxo-ferric heme intermediates can be obtained from the recent porphyrin models developed by Naruta and coworkers [207–209]. High-quality rR spectra of oxy-complexes and both low-spin end-on and high-spin side-on peroxo-ferric complexes have been measured in acetonitrile and in methanol at low temperatures (208 K) or in frozen solutions at 77 K. However, the proximal ligand to the iron in these model complexes is imidazole, and hence they can be considered as appropriate models for the oxygen activation intermediates in peroxidases, rather than the P450 enzymes. Interestingly, both Fe–OO and O–O modes have been observed in these complexes, contrary to the peroxo- and hydroperoxo-ferric complexes in myoglobin, where the O–O stretch mode was not detected in rR spectra [201, 210].

While early X-ray crystallographic investigations did not fully appreciate the *in citu* reduction of the prosthetic groups of metalloproteins, it is now clear that the X-ray beam, particularly from intense synchrotron sources, can efficiently add electrons to the system. An important advance in protein X-ray crystallography was achieved when cryoradiolytic reduction of the oxy-complex in CYP101A1 was intentionally used [92]. The unavoidable reduction of the heme complexes during data collection at cryogenic temperatures was carefully monitored and controlled by combining data obtained on multiple crystals [181, 211]. Following this approach, the first well-characterized structures of the unstable Compound 0 in horseradish peroxidase [211] and in CPO [212] were realized, and a high-resolution structure of the Compound 0 (peroxo-intermediate) in myoglobin was obtained [213]. The latter structures provide good experimental data on the O–O and Fe–O bond lengths in the protein hydroperoxo-ferric complexes.

3.7 Reactivities of the Peroxo States: O–O Bond Scission Versus Peroxide Dissociation

A second protonation of Compound 0 at the distal oxygen atom reduces the O–O bond order to zero and results in immediate scission and departure of a water molecule [214]. In cryoradiolytic experiments, Compound 0 is stable below the glass transition temperature, typically 180–190 K. This suggests that the second proton delivery requires sufficient mobility and diffusion of solvent molecules, with the potential relaxation of the protein matrix to a new conformation. Experiments with native CYP101A1 and the D251N mutant proved that at higher temperatures, Compound 0 disappears with formation of Compound I **[6]** (Fig. 3.1) and concomitant product formation [185]. For the CYP101A1 T252A mutant, where the native proton transfer mechanism is perturbed, the dissociation of peroxide with no product formation is the dominant path of Compound 0 decomposition. The latter reaction is considered as the main source of reactive oxygen species in the poorly coupled P450 systems. In general, the coupling efficiency measured by the ratio of the product molecules formed per NADPH molecule consumed can be very different for the same cytochrome P450 with different substrates. Efficient

proton delivery requires specific positioning and stabilization of water molecules in the vicinity of the dioxygen moiety, which can be significantly perturbed by variations in the structure of the substrate.

The chemical mechanisms describing the hydroxylation of unactivated substrates most assuredly involves the Compound I intermediate state generated after O–O heterolysis following second proton transfer as described above. This is not necessarily the case for reactions involving carbon–carbon bond scission. For instance, in the case of CYP19 (aromatase)-catalyzed androstenedione (AD) metabolism, it has been a longstanding question as to whether the conversion of 19-oxo-AD to estrone by CYP19A1 occurs via the classic higher valence Compound I intermediate that operates in the normal hydroxylation cycle, or via the precursor peroxo-anion (Compound 0) intermediate. This is shown schematically in Fig. 3.7.

Evidence supporting both hypotheses is present in the literature [215–218], as the availability of a nearby proton for abstraction makes both a radical and nucleophilic mechanism plausible. In the first experiments with human CYP19A1 self-assembled into Nanodiscs, we discovered that when the ferrous-oxy complex was radiolytically reduced in the presence of AD, the peroxo state formed and stabilized at 77 K was the anionic form rather than the protonated hydroperoxo that had been seen in all previous P450s investigated [194]. This suggested that there was perhaps a different hydrogen-bonding configuration provided by active site water molecules in this P450. However, experiments monitoring the conversion of AD to 19-hydroxy-AD in an EPR-annealing experiment revealed a kinetic solvent isotope effect of greater than 3.5, suggesting one or more protons were involved in product formation from AD [219]. More recent EPR results demonstrated that when the substrate is 19-oxo AD, the immediate precursor to the carbon–carbon lyase reaction, the species stabilized at 77 K after radiolysis, and before product formation by CYP19A1, is the protonated (hydroperoxo) intermediate,

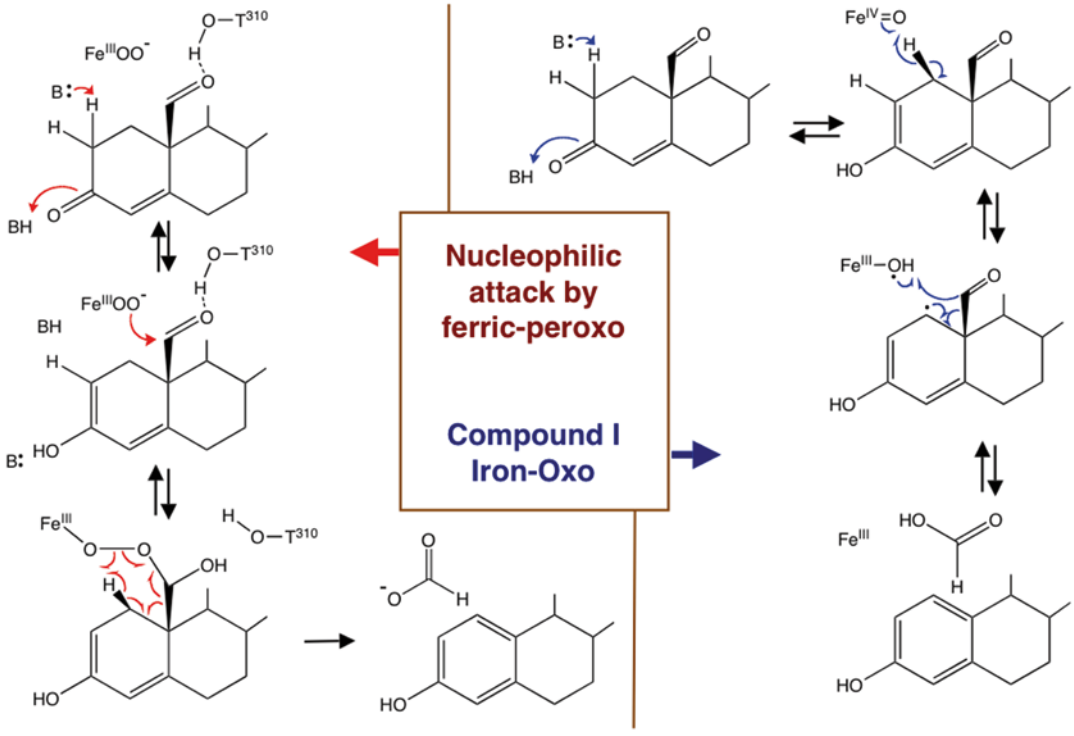

Fig. 3.7 Two alternative mechanisms of C–C bond scission in CYP19A1

as one would expect for a normal Compound I-mediated reaction. There are thus subtle differences in the active site structure that dictate a key variability in distal pocket hydrogen bonding and proton transfer, but it appears that Compound I is the "active oxygen" leading to C–C bond cleavage and aromatization of the A-ring. Exactly the opposite is true in the case of CYP17A1, where a nucleophilic reactivity of Compound 0 appears to be operating. This will be discussed further in the following section.

A carbon–carbon bond cleavage required for conversion of the pro-drug nabumetone to the active form is also catalyzed by the peroxo-ferric intermediate of human CYP1A2, as reported based on a thorough study comparing the activities of several human cytochromes P450 [220]. Only CYP1A2 and CYP3A4 (CYP2B6 with significantly lower efficiency) supported the C–C cleavage reaction with nabumetone and 3-hydroxy-nabumetone as substrates. In addition, C–C cleavage did not proceed when the peroxide shunt pathway with cumene hydroperoxide was used instead of NADPH supported catalysis. However, the NADPH-supported hydroxylation of nabumetone in reconstituted systems and in commercial Supersome® preparations was observed with almost all the isozymes, the most efficient being CYP2C19, CYP2B6, and CYP3A4. These observations suggest that the unprotonated peroxo-ferric intermediate is the main catalytic species for C–C bond cleavage in this system.

3.8 Compound I as the "Active Oxygen" in Alkane Hydroxylations

Despite the great variety of chemical transformations catalyzed by cytochromes P450, the vast majority of them are undoubtedly driven by Compound I. This ferryl-oxo intermediate with a π-cation radical delocalized on the porphyrin is a very reactive species. All attempts to observe this species in a P450 system using atmospheric dioxygen have so far failed. However, important spectroscopic characterization and reactivity measurements have been obtained by using the peroxide shunt pathway **[3] →[6]** in Fig. 3.1. In this approach, which bypasses the dioxygen reduction process, rapid mixing of the ferric heme enzyme with peroxides or peroxy acids such as meta-chloroperoxybenzoic (*m*-CPBA) can generate the Compound I intermediate directly [221–223]. Unlike the usual P450 pathway of oxygen activation, where two electrons and two protons have to be channeled to the dioxygen via coordination to the heme iron and proton delivery pathways, the peroxide pathway benefits from the fact that peroxides or peroxyacids already have the two electrons and protons on the dioxygen moiety. The role of the enzyme in this case is the efficient rearrangement of the proton from the proximal oxygen atom, which forms the transient coordination bond with the heme iron, to the distal oxygen to facilitate heterolytic scission of the O–O bond and thus create the same Compound I, as happens in the normal catalytic pathway of horseradish peroxidase [224–226]. However, in general, the cytochromes P450 are inefficient peroxidases or peroxygenases, and the yield of Compound I by this pathway is low. Thus the first experiments devoted to revealing this intermediate via stopped flow realized a yield of ~10% or less [221–223]. This low level of protein made it all but impossible to obtain detailed structural and spectroscopic characterization of the Compound I in cytochromes P450. Until recently, the only way to address experimentally the physico-chemical and functional properties of this intermediate was via model porphyrin systems [10, 227–230] or by analogy to other closely related thiolate-ligated heme enzymes such as CPO [231–233] and peroxygenases [234, 235], for which Comopund I is much more stable.

This situation changed with the work of Rittle and Green who achieved a breakthrough on the peroxide pathway by radically improving the purification protocol for thermostable CYP119 from the extremophile archae *Sulfolobus acidocaldarius* [236–238]. Careful multistep removal of endogenous substrate analogs from the purified, heterologously expressed protein, which hampered earlier studies [222], allowed them to dramatically increase the yield of Compound I in a stopped-flow reaction with *m*-CPBA, reach-

ing a conversion of greater than 75% [236]. This made possible high-precision UV–vis spectra to quantitate the reaction kinetics, which in turn provided the necessary information for the preparation of highly concentrated samples for EPR and Mössbauer spectroscopy. The UV–vis spectra of Compound I confirmed the main features of the ferryl-oxo π-cation radical known from the earlier experiments: a broad Soret band at 367 nm and a pronounced charge-transfer band at 690 nm. The EPR spectrum of CYP119 Compound I [236] had a different shape as compared to that previously reported for CPO, another thiolate-ligated heme protein [239]. Fitting of both spectra to the S = 1 Fe(IV)-oxo unit coupled with S = 1/2 porphyrin radical resulted in a higher ratio of the exchange coupling (J) to zero-field splitting (D) for CYP119 ($J/D = 1.3$) than in CPO ($J/D = 1.02$) [236]. The higher J value in CYP119 was tentatively attributed to either a higher spin density on the thiolate sulfur atom or a shortened Fe–S bond. The Mössbauer parameters measured for the CYP119 Compound I were more similar to those of CPO [239], with the isomer shift $\delta = 0.11$ mm/s (0.13 mm/s for CPO) and quadrupole splitting $\Delta E_Q = 0.96$ mm/s (0.90 mm/s for CPO). These parameters also correspond to the ferryl-oxo S = 1 unit exchange coupled to the porphyrin radical (S = 1/2).

The functional competence of this Compound I intermediate was confirmed in fatty acid hydroxylation assays using a double-mixing stopped-flow technique. After premixing CYP119 with *m*-CPBA and incubating for 100 ms, the reaction mixture containing 35–40% of Compound I was rapidly mixed with solutions of the substrates at various concentrations at 4 °C. The kinetics of the reactions were monitored spectroscopically and the product yield was verified by gas chromatography ([236] and supporting online material). The observed apparent rates were very high, up to 220 s^{-1} for lauric acid, with the rate constants varying from 4.4×10^4 to 1.1×10^7 $M^{-1}s^{-1}$ for hexanoic and dodecanoic (lauric) acids, respectively. In addition, the kinetic isotope effects (KIE) for these reactions measured experimentally with protonated and perdeuterated substrates strongly depended on the chain lengths of the fatty acids, varying from 12.5 for hexanoic acid to 1.0 for lauric acid. This disappearance of the KIE for the fast-reacting substrate is explained by strong masking of the isotope effect by tight substrate binding and rate-limiting unproductive substrate dissociation for lauric acid. The true isotope effect value can be measured only when substrate binding is at rapid equilibrium, as demonstrated in [236] and supporting material. Thus, the high-unmasked KIE strongly confirms the catalytic competence of the Compound I obtained in CYP119 by rapid mixing with *m*-CPBA and the kinetic parameters expected for the hydrocarbon hydroxylation via a hydrogen-abstraction mechanism [11, 227].

Recently, the same improved multistep purification approach proved to be critically important for the generation of high populations of Compound I in another cytochrome P450, $P450_{ST}$ [238]. Following similar experimental protocols, Green and his group were able to trap Compound I in a high concentration and to measure its EPR and Mössbauer spectra. The results were similar to those measured for CYP119 [236]. Mössbauer spectra could be fitted well with an isomer shift $\delta = 0.12$ mm/s and a quadrupole splitting $\Delta E_Q = 0.85$ mm/s, and $J/D = 1.3$ obtained from EPR spectra that were the same as for CYP119 [238].

The oxygen-rebound mechanism of hydrocarbon hydroxylation catalyzed by Compound I presumes formation of the transient heme intermediate equivalent to Compound II following hydrogen abstraction from the substrate. In this case, the iron-oxo unit is protonated, and the electron fills the π-cation radical of the porphyrin [236, 240]. The critical importance of thiolate ligation in P450 catalysis was evaluated by recent work from the Green group [241]. By direct measurements of pK_a of the Compound II of CYP158, they estimated and compared the relative contributions of redox potential and proton affinity to the thermodynamics of hydrogen atom abstraction by Compound I in cytochrome P450, CPO, and nitric oxide synthase. The key difference between histidine-ligated peroxidases and thiolate-ligated P450 enzymes is the large shift of the pK_a of Compound II from ~3.5 in the for-

mer to ~12 in the latter, due to the much stronger electron-donating abilities of a thiolate than a histidine. At the same time, the large contribution from the strong proton affinity term makes the redox potential term low enough to prevent fast inactivation of this catalytically active intermediate by intra-protein electron transfer and reduction to Compound II [242, 243]. The same effect of the thiolate proximal ligand was observed by Hoffrichter and Groves in a thiolate-ligated peroxygenase [235]. Thus we have for the first time a clear mechanistic rationale as to why the cytochromes P450 utilize cysteine as the axial ligand to the iron [235, 242, 243].

3.9 Bleed Points of Inefficiency: Uncoupling Pathways in the Cytochromes P450

The key characteristics of enzymatic catalysis are the maximum rate of product formation given by V_{max} or k_{cat}, and the substrate-binding constant, or Michaelis constant K_m. For comparison of different enzymes and/or substrates, the efficiency of the enzyme is characterized by the ratio of these two parameters. For cytochromes P450, these parameters also can be used as the essential quantitative measures of their ability to metabolize xenobiotic compounds or to synthesize their specific products. The case of P450 catalysis, however, is complicated by the consumption of redox equivalents and the nature of atmospheric dioxygen as a reactant. The ideal stoichiometry of P450 catalysis requires one NAD(P)H and one O_2 molecule to make one molecule of product. This rarely happens in reality. In addition to product formation, a fraction of oxygen is released in the form of superoxide after one redox transfer event, as peroxide after two-electron reduction, or as water after four-electron reduction, as shown in Fig. 3.1. Superoxide and hydrogen peroxide belong to a class of compounds termed "reactive oxygen species" or ROS. A comprehensive review on ROS production by P450 summarizes the main mechanisms as well as the implications of the release of these potentially toxic products [149]. Other side reactions, such as formation of protein radicals, covalent coupling of the heme to the protein or to active radical products, heme loss, or accumulation of the inactive P420 form, can also be considered as the consequences of uncoupling and have been reviewed elsewhere [244].

Oxygen activation in cytochromes P450 is a multistep process with several branching points. As seen from Fig. 3.1, there are at least three steps where the reaction flow can partition between productive and unproductive pathways. The first one is the oxy-complex, which can decompose with dissociation of superoxide if the second electron transfer is not efficient or simply not fast enough. The second branching point is dissociation of the hydroperoxo-anion, if the second protonation is not accomplished. The third one is unproductive reduction of compound I with consumption of a second molecule of NAD(P)H, which can occur if a productive catalytic reaction with the substrate does not happen. All these branching points are essentially kinetic, and the result at each step is determined by the corresponding rate constants. Under steady-state conditions, the overall degree of uncoupling is determined by the ratio of the reaction flux along the productive pathway **[3]→[4]→[5]→[6] →[7]** in Fig. 3.1 and the sum of fluxes along the unproductive pathways **[4]→[2], [5]→[2],** and **[6]→[2].** Partitioning at each branch point is proportional to the absolute microscopic rate constants leading out of the intermediate. For progress from the oxy-complex, the fraction of the overall reaction flux that follows the uncoupling pathway is proportional to the autoxidation rate k_{42}, while the fraction of oxy-complex reduced to the peroxo-ferric state is proportional to k_{45}. Thus, the fraction of the reaction flux following the productive pathway, or the coupling ratio at the level of the oxy-complex, is $k_{45}/(k_{45}+k_{42})$. The second branch point lies with the peroxo states, where it is possible to release a twice-reduced dioxygen to regenerate the ferric prosthetic group. Similarly, a coupling ratio taking together the peroxo- and hydroperoxo intermediates as **[5]** is determined by the ratio of protonation rates and the rate of peroxide dissociation, $k_{56}/(k_{56}+k_{52})$. The third uncoupling point in the P450 reaction cycle centers on the Com-

pound I intermediate **[6],** which can be reduced by two additional electrons to form water. Since the overall stoichiometry is then four electrons and four protons added to one dioxygen yielding two water molecules, this has been termed the oxidase pathway. From the scheme in Fig. 3.1, the overall partition coefficient for the oxidase branch point is given by $k_{67}/(k_{67}+k_{62})$. Overall, the experimentally observed uncoupling is proportional to the product of these three fractions, while the absolute rates of substrate conversion to product and of NAD(P)H and O_2 consumption, in most cases, depends on the rates of the first and second electron transfers, protonation of the dioxygen moiety, and the catalytic step, although in some cases substrate binding and product release may also be rate limiting.

In reality, many of these individual rate constants are not known and difficult to measure. In most cases, there is no single and well-defined rate-limiting step in the overall catalytic cycle of the cytochromes P450 and thus several intermediates are present at any one time. Early attempts to monitor the steady state of P450 catalysis usually focused on the oxy-ferrous intermediate, which was observed experimentally during turnover using optical absorption spectroscopy [157, 158, 245]. The rate of autoxidation k_{42} can be measured separately with high precision, as described earlier in this review and in previous publications and review articles [36, 82, 87, 246–248]. The rate of the second electron transfer to the oxy-complex, k_{45}, which competes with autoxidation, is more difficult to probe at ambient conditions due to autoxidation and possible rate-limiting interactions with redox partners. Successful examples are represented by stopped-flow studies with various concentrations of Pdx and CYP101A1 [154, 155]. Measurements of the peroxide dissociation rate, k_{52}, and oxidase uncoupling rate, k_{62}, are even more difficult because the steady-state concentrations of the key intermediates **[5]** and **[6]** are exceedingly small. To our knowledge, no independent reports of these rate constants at ambient conditions are available. However, the steady-state rates of peroxide production and water production via the oxidase uncoupling channel are known for many cytochromes P450 in reconstituted systems, so the fractional partition coefficients at the peroxide **[5]** and Compound I **[6]** branching points can be estimated.

Partitioning at these three uncoupling branch points and the corresponding rates determine the overall efficiency of substrate turnover. This depends on various factors, which include the substrate structure and its positioning inside the substrate-binding pocket, the efficiency of proton delivery to the coordinated dioxygen via the hydrogen-bonded network of several protein groups together with strategically placed and conserved water molecules, and the efficiency of electron transfer from the protein redox partner. Clearly, changes of each of these factors may significantly affect the result of oxygen activation and change the partitioning between productive and unproductive pathways. These two pathways may be described as oxygen activation for either the oxidative transformation of organic substrates or the production of peroxide and water. For the most efficient cytochromes P450, such as CYP101A1 and CYP102A1, where catalysis via the productive pathway with optimal substrates is realized with almost 100% efficiency, even small variations in the substrate structure or single-point mutations at the active center result in significant uncoupling and redistribution of the reaction flow towards peroxide production. For those cytochromes P450 that are significantly uncoupled (either *in vitro or in vivo*), the same mutations and/or substrate variations may be favorable for the increase or the productive consumption of NAD(P)H and O_2. The same is true for inefficient metabolism of nonnative substrates by the wild-type enzymes, where mutations may significantly improve the rate of oxygen activation and coupling to the productive pathways. Multiple mutations at the substrate-binding pocket not only can drastically change the regio- and stereospecificity of substrate binding but also can be engineered to alter uncoupling and extend the range of chemical transformations of nonnative substrates catalyzed by cytochrome P450. These results have been extensively reviewed for the self-sufficient CYP102A1, which is considered as the most promising cytochrome P450 for bio-

engineering and synthetic biology purposes [27, 249, 250].

Uncoupling at the oxidase branch point has been observed in microsomes by comparing the rates of NADPH and O_2 consumption with a natural substrate with those of a non-metabolized analog, as in the case for CYP21 in bovine adrenocortical microsomes [38]. Here, the 2:1 NADPH/O_2 stoichiometry was correctly assigned to the oxidase uncoupling pathway. Similar observations were reported by Ullrich [39, 251] using perfluorinated substrate analogs to prevent productive reactions of Compound I. They also observed a 2:1 NADPH/O_2 consumption stoichiometry. Later Coon and coworkers documented both productive and unproductive pathways, including peroxide and oxidase uncoupling, using purified liver microsomal cytochrome P450 and demonstrated that the results strongly depend on the substrate [252]. A review of the overall stoichiometry of coupled and uncoupled P450 reactions has been provided by Zhukov and Archakov [253, 254].

The availability of X-ray structures for CYP101A1 [32, 33, 255–259] made possible a detailed analysis of uncoupling in P450 catalysis. The hydroxylation of the natural substrate 1*R*-camphor by wild-type CYP101A1 is very fast (k_{cat} up to 35 s^{-1}) and almost 100% coupled, with an NADH/product ratio of ~1.02–1.03. This provides an excellent reference system for systematic study of the relative importance of essential features of the enzyme active site as well as substrate structural variations for overall catalysis. A series of CYP101A1 mutants has been generated based on the available X-ray structures with the goal of deciphering the structural determinants of efficient substrate hydroxylation [23, 24, 260–268]. Various substrate analogs were also employed to explore the regio- and stereospecificity of chemical transformations catalyzed by CYP101A1 together with the rate and efficiency of steady-state turnover [23, 261–263, 265]. Taken together, these works revealed a great variability of both rates and coupling efficiencies that depend on both on single mutations and variations of the substrate structure.

The discovery of the critically important acid–alcohol pair D251-T252 in CYP101A1 was very important from a mechanistic point of view. The dramatic effect of a D251N mutation, which resulted in a 50–100-fold slowing of the product-formation rate, without loss of the efficiency of NADH consumption, and the same ~95% coupling as in the wild-type enzyme [267], clearly indicated the gate-keeping role of this residue in proton delivery. Its role was later confirmed by kinetic solvent isotope effect (KSIE) and proton inventory measurements [269]. Impaired proton delivery in the D251N mutant as the main cause for slow turnover was also confirmed by directly measuring the rate of the first electron transfer from Pdx, which was even faster than in the wild-type enzyme [270]. In addition, significant acceleration (5–10 times) of NADH consumption and camphor hydroxylation observed at moderately acidic pH (5.5–5.0) also supported protonation as the strongly rate-limiting step in the CYP101A1 D251N mutant [267]. The effect of the salt link between D251 and K178 was tested separately by mutating this residue to glutamine, K178Q [267]. This mutant was highly coupled and only moderately slower than the wild-type enzyme, implying that the position of the side-chain of D251 is not the main factor determining the overall turnover efficiency of CYP101A1. In contrast, the T252A mutation of the neighboring residue uncoupled hydroxylation catalysis by ~95% with no inhibition in NADH consumption, efficiently channeling redox equivalents into peroxide production [271, 272]. The essential role played by the alcohol side-chain of T252 in oxygen activation was confirmed by the high activity and 81% coupling of the T252S mutant [272]. The importance of these two residues in the CYP101A1 mechanism has been analyzed in great detail using X-ray structures of the oxy-complexes of these mutants [74, 92]. The structures of oxy-complexes reveal important conformational rearrangements of the I-helix, with reorientation of the T252 side-chain opening the cleft between T252 and G248, and appearance of two new well-resolved water molecules that most likely represent the main proton delivery channel [74, 92, 93, 95]. The same opening in the I-helix and same water molecules were

also observed in the structure of the cyanide complex of CYP101A1 [273] and the highly similar CYP101D1 [274], possibly due to very similar geometries of the Fe–O_2 and Fe–CN^- complexes with an angle of ~125° between the ligand axis and heme plane, and similar H-bonding properties.

The recent discovery and crystallization of other members of the CYP101 family, CYP101D1 [274, 275] and CYP101D2 [276], opened additional means to probe the finely tuned and highly efficient mechanism of oxygen activation. Both CYP101D1 and CYP101D2 bind camphor in the same orientation as CYP101A1 and catalyze the same hydroxylation with similar high rates (1000–2000 min^{-1}) and almost 100% efficiency [274, 277]. Despite the same activity towards the same substrate, there are structural and functional differences between these three isozymes that provide a better understanding of the essential (and not essential) features for optimal P450 catalysis. Mutations of the acid–alcohol pair residues D259N and T260A in CYP101D1, analogous to D251N and T252A in CYP101A1, had the same effect: Little or no activity in the Asp/Asn mutant and highly uncoupled NADH consumption in the Thr/Ala mutant in both proteins [274]. Critical variations in CYP101D1, as compared to CYP101A1 (where G180 replaces the homologous K178, D182 is used instead of N184 and A366 in CYP101D1 replaces L358 in CYP101A1) may have changed the functional properties with respect to interactions with the redox partner Pdx and/or protonation/substrate binding. When these mutants were introduced into CYP101A1 to check for the functional implications of these residues using the native redox partner Pdx [275], the single mutants L358A and K178G had little effect on the activity or structure of CYP101A1. However, the double mutant L358A/K178G had a tenfold slower rate of NADPH consumption than the wild type due to the mostly low-spin state even in the presence of camphor. The addition of 400 mM K^+ converted the double mutant protein to the high-spin form and diminished the difference in steady-state NADPH turnover. The crystal structure of the cyanide complex of the mutant CYP101A1 shows the same structural changes as in the wild type, including the key water molecules in the I-helix cleft, indicating that the proton delivery pathway is not perturbed by these mutations [275].

Homology analysis revealed that the acid–alcohol pair in the I-helix is a common feature in the great majority of cytochromes P450, although some deviations are evident. In the CYP51 class the semi-conserved acid side-chain (D251 in CYP101A1) is replaced by a histidine [278]. In the rat CYP51 enzyme, the mutation H314D resulted in a sevenfold lower 14-demethylase activity [279]. In some cytochromes P450 the alcohol side-chain from threonine or serine is replaced by alanine, as in P450eryF (CYP107A1) and CYP158A2. Based on the X-ray structural studies of these two enzymes, the concept of substrate-assisted catalysis was proposed as an alternative to the missing side-chain of Thr/Ser [80, 280]. The functionally important water molecules forming the proton delivery pathway are stabilized at the proper position close to the coordinated dioxygen by hydrogen bonding to the substrate hydroxyl group instead of the alcohol side-chain [73, 80, 280]. In CYP107A1 the mutants A245S and A245T have lower activity and higher uncoupling to produce peroxide, which was attributed to perturbations in positioning of the functionally important waters [73, 280–282]. However, the A245T mutant gained the ability to catalyze the hydroxylation of alternative substrates such as TST [283] and 7-benzyloxyquinoline [284], providing further evidence in support of the general importance of this alcohol side-chain for the P450 catalytic cycle. Other examples of naturally occurring variations in the acid–alcohol pair include the replacement of threonine by asparagine N242 in CYP176A1 [285] and N240 in P450 OxyB [286], glutamine Q230 in CYP165D3 [287], and proline P237 in CYP134A1 [288]. Still, these enzymes perform the usual P450 chemistry with oxygen activation. In contrast, in P450 peroxygenases, which do not follow the regular P450 oxygen activation cycle, but rather react via the peroxide shunt mechanism, both acid and alcohol residues are replaced by other amino acids, such as V245-A246 in CYP152A1 [289] and I248-A249 in CYP152L1 [290].

Both the semi-conserved acid and alcohol residues were mutated in other cytochromes P450 in order to understand the importance of these features for P450 catalysis. In CYP102A1 the T268A mutation similar to T252A in CYP101A1 resulted in a decrease in both NADPH consumption and product turnover rates with several substrates [291, 292], but not with pentadecanoic acid, for which coupling was maintained at the same level as in the wild-type enzyme [293]. The high coupling with some substrates and strong dependence on the chain length of the fatty acid (34 vs. 10% for C_{12}, 88 vs. 74% for C_{14}, 88 vs. 89% for C_{15}, and 93 vs. 21% for C_{16}, in the wild type vs. T268A mutant correspondingly) clearly demonstrates variations due to the packing of the substrate in the overall efficiency of P450 turnover, as well as the difference caused by the Thr268 mutation. These results again stress the critical role of the substrate in the modulating water access to the active site and thus the overall catalytic efficiency defined as the ratio between productive and non-productive pathways. At the same time, the absolute rates of NADPH consumption also strongly depend on the T268A mutation in CYP102A1 [292]. The general conclusion based on the comparison of the wild-type CYP102A1 and T268A mutant is that the presence of this threonine is not absolutely essential for hydroxylation, but it is certainly important in providing an efficient proton delivery pathway with most substrates [293].

CYP176A1 (P450cin) is a close analog of CYP101A1, with its natural substrate cineol also being similar to camphor. However, in wild-type CYP176A1 the conserved threonine residue is replaced by asparagine N242 [294]. The fact that this enzyme is nevertheless catalytically competent in cineole hydroxylation with almost the same efficiency as CYP101A1 in camphor hydroxylation (rate of NADH consumption 950 min^{-1} and coupling ~80%) [295] attracted the attention of several research groups and inspired a detailed analysis of its structure and mechanistic issues [285, 295–300]. Mutation of the unusual N242 to a threonine (N242T) [295] or alanine (N242A) [298] resulted in a moderate decrease in coupling, 54 and 72% respectively, as compared to 80% in the wild-type enzyme. At the same time significant (sixfold and fourfold) decreases in the absolute rates of NADH consumption were observed with these mutants. Both results are very different from the very strong uncoupling with fast NADH consumption in the CYP101A1 T252A mutant. Attempts to test in CYP176A1 the substrate-assisted mechanism of oxygen activation found for CYP107A1 (P450eryF), where the hydroxyl group of the substrate replaces the threonine side-chain in stabilizing the proton delivery pathway, proved inconclusive [298]. Unlike in CYP107A1, where replacement of the key hydroxyl group of the substrate by a ketone inhibited hydroxylation by more than 100-fold [301], similar modifications of the native substrate cineol produced only a moderate decrease in activity and coupling [298]. Therefore, no clear understanding of the predominant structural features affecting proton delivery and efficiency of oxygen activation came out of these mutation studies. Alternatively, a comparison of CYP176A1 and CYP101A1 suggests the presence of multiple pathways for protonation of the dioxygen moiety, which compensate to a certain extent for the loss of an important functional group in the active site.

The same conclusion may be made based on the effect caused by the T252N mutation in CYP101A1, which mimics the N242 residue in CYP176A1. This was done to test the ability of asparagine to replace the key T252 [302]. As in CYP176A1, the Asn252 mutant in CYP101A1 demonstrated efficient camphor hydroxylation with an overall 42% coupling determined as the ratio between the rates of product formation and NADH consumption. The main difference between the wild-type and mutant protein was an almost 20-fold lower affinity for camphor binding in the T252N mutant. This work provides one more example of the great flexibility and robust design of active centers in cytochromes P450, which remain functional despite various mutations.

Both the acid and alcohol residues, Glu318 and Thr319, have been mutated in CYP1A2 in order to evaluate the mechanism of oxygen activation in this cytochrome P450 [303, 304]. Sur-

prisingly, in some cases the mutations improved the overall coupling of methanol oxidation from 9% in the wild-type protein to 16% in the E318A mutant, and even to 40% in the T319A mutant [303], despite the slower product-formation rates, 25% and 39% of the rate of 4.4 min^{-1} observed in the wild-type enzyme. In addition, no H_2O_2 was detected with the E318A and T319A mutants, with most of the uncoupling attributed to the oxidase (water) channel. In contrast, the same E318A mutant was almost inactive when 7-ethoxycoumarin was used as a substrate by the same authors, whereas the T319A mutant was even more active than wild-type [304]. These observations led the authors to suggest that the role of the conserved threonine in oxygen activation may be different, or at least not as critical, in CYP1A2. However, later studies demonstrated that even changes in the buffer composition, pH, and temperature could significantly change uncoupling by a factor of three [305].

In CYP2B4 the T302A mutation also significantly inhibits N-demethylation of benzphetamine and hydroxylation of cyclohexane with the rates decreasing 20-fold [56]. The steady-state NADPH consumption rates are also significantly slower when the T302A mutant is used, but H_2O_2 production is sometimes even higher. This fact can be interpreted as a slower proton delivery in the mutant enzyme and a longer lifetime of the peroxo- and hydroperoxo-ferric intermediates, with a predominantly dissociative unproductive pathway favored over productive protonation and Compound I formation. This hypothesis is also consistent with the tenfold increase in the rate of cyclohexane carboxaldehyde deformylation, apparently catalyzed by the peroxo-ferric intermediate, and not by Compound I [56]. The peroxo-anion reaction with aldehydes to give a peroxyhemiacetal intermediate, followed by homolytic scission of the O–O bond, was also invoked to explain the mechanism of substrate-assisted heme destruction and the faster rate of heme loss in the T302A mutant than in the wild-type protein [306]. The activation of the conversion of a *p*-hydroxybenzene derivative to a hydroquinone caused by the T303A mutation in CYP2E1 was attributed to a more efficient catalysis of the *ipso*-substitution reaction by the hydroperoxo-ferric intermediate in the mutant enzyme due to perturbed protonation and loss of the Compound I pathway [307]. Interestingly, the effects observed in these works strongly depended on the substrates, with the k_{cat} increase caused by T303A in CYP2E1 varying from 1.1 with 4-fluoro phenol to 31 with 4-bromo phenol [307], indicating the importance of the electron-withdrawing halogen substituents. At the same time, the authors noted that this variation may indicate a difference in the predominant mechanism in the wild-type and CYP2E1 T303A mutant, consistent with the concept of multiple "active oxygen" intermediates in P450 catalysis as recently reviewed [308, 309].

Substrate dependent uncoupling is clearly manifested in CYP3A4, which can bind up to three substrate molecules such as TST [82, 140, 310]. Using global analysis of multiple experimental data sets measured under identical conditions, it was possible to resolve the fractional contributions of intermediates with one, two, or three TST molecules bound to CYP3A4 in the overall NADPH consumption and product formation rates. The first binding of steroid substrate to the remote binding site did not result in formation of product, but increased the NADPH consumption rate by a factor of four, likely due to stabilization of the oxy-complex [82] and more efficient second electron transfer. Binding of the second substrate molecule caused almost complete shift to the high-spin state and resulted in product formation at almost the maximal rate. At the same time, the NADPH consumption rate also increased, so that the coupling in this case was only 5%. Binding of the third substrate did not change the rate of product formation, but improved coupling to ~13% [140]. This nontrivial dependence of the rate and efficiency of CYP3A4 catalysis on the substrate concentration demonstrates the complexity of the mechanism of oxygen activation with many parameters determining the overall outcome.

The substrate dependence of the oxidase uncoupling channel in CYP3A4 provided more information about productive and non-productive pathways and the role of the lipid bilayer in the overall efficiency of TST hydroxylation [310].

Even in the absence of substrate the oxidase uncoupling channel accounted for almost 20% of the total oxygen consumption, indicating possible formation of Compound I in substrate-free CYP3A4. At saturating TST concentrations, the absolute rate of water production increased from 8 to 52 min^{-1}, or ~25% of the total oxygen consumption, with total peroxide production decreasing from 80 to 65%, and the product-formation rate to 25 min^{-1}. These results provide a rough estimate of the partitioning between productive (TST hydroxylation, **[6] ->[7]** Fig. 3.1) and unproductive (water production, **[6] ->[2]** Fig. 3.1) pathways at the Compound I level. In this scheme, the ratio $k_{67}/k_{62}=0.5$ gives an estimate of the relative probability of a successful catalytic event as Compared to the unproductive decay of Compound I with TST as a substrate. The same rate of oxidase uncoupling was observed with bromocriptine as a substrate [310], although the product formation rate was slower. The presence of 30% anionic lipid 1-palmitoyl-2-oleoyl-phosphatidylserine improved overall coupling and facilitated product formation for TST and bromocriptine by a factor of 1.5–2.

Important results on the specific mechanisms of uncoupling in CYP101A1 have been obtained by Makris et al. [189] by comparison of the G248T and G248V mutants with the wild-type protein. The second proton delivery was significantly inhibited in both mutants, so that the overall NADH consumption rate decreased by factors of 4 and 13, respectively. In addition, the coupling efficiency (ratio of the product formation rate to the NADH consumption rate) fell from 98% to 74% and 28%. Additional information has been provided by comparison of the steady-state kinetic parameters measured in H_2O and D_2O. Increased uncoupling in D_2O was observed in all cases, but to a different extent, with the ratio of the product formation rate constants ranging from 1.1 in the wild-type protein to 1.75 in the G248V mutant. This variation indicates that the second proton transfer is at least in part rate limiting in the CYP101A1 catalytic cycle; otherwise, there would be no apparent difference in the steady-state kinetic parameters in H_2O and D_2O. In both the G248T and G248V mutants, the steady-state rate of NADH consumption was slightly higher in D_2O, despite the slower rate of product formation in deuterated solvent. This is the result of impaired protonation of the peroxo- or hydroperoxo-ferric intermediate in D_2O and redistribution of the reaction flux towards H_2O_2 production.

Because the productive pathway of substrate metabolism includes protonation steps, it depends on the solvent H/D composition and is slower in D_2O than in H_2O. In contrast, the autoxidation and peroxide dissociation rates are not as strongly proton-dependent and hence are less affected by solvent composition. Therefore, P450 catalysis in D_2O is usually slower and less efficient (more uncoupled) than in H_2O. The observation of an inverse isotope effect is usually interpreted as an indication of some mechanistic change or different catalytic pathway. This is the case in the C–C cleavage reaction catalyzed by CYP17A1 [311]. The hydroxylation of pregnenolone at C17 proceeds through the common P450 pathway with Compound I as the catalytic intermediate and thus is proton dependent. In agreement with this mechanism, the KSIE measured for this step is small (~1.3), as is seen in other cytochromes P450. However, a large inverse isotope effect with $k_H/k_D=0.39$ for the second lyase step with the 17-hydroxypregnenolone as a substrate, cannot be rationalized using the same catalytic pathway. Analysis of the protonation-dependent and protonation-independent pathways (Fig. 3.1) supports the alternative mechanism of lyase catalysis via the unprotonated peroxo-ferric intermediate, first proposed by Akhtar [216] and still debated in the literature [312]. Unlike the regular P450 pathway via Compound I, this reaction goes directly from [**5**] to product and does not require proton delivery. A productive pathway in such mechanism is not expected to show any KSIE. Alternatively, the peroxo-ferric intermediate can be protonated and form a hydroperoxo-ferric intermediate, which can then dissociate without product formation and contribute to proton-dependent uncoupling. Unlike normal P450 catalysis via proton-dependent formation of Compound I, for the peroxo-driven pathway product formation is not proton-dependent, while

uncoupling involves protonation of peroxide and dissociation of H_2O_2. As a result, for peroxo-ferric driven catalysis of the lyase reaction in CYP17A1 an inverse KSIE is expected, exactly as observed experimentally [311].

3.10 Summary

The complex multistep mechanism of oxygen activation in P450 represents a finely orchestrated process in which contributions from multiple players have to be delivered timely and in a proper order. Oxygen activation is necessary for accelerating reactions in which dioxygen as a free molecule in gas or solution would never engage because of very high activation barriers. Considering dioxygen as a reagent, the P450 cycle (Fig. 3.1) can be viewed as an oxygen activation process with multiple possible outcomes. The most important for living organisms are the two pathways that result in oxidative transformations of organic molecules catalyzed by heme-oxygen intermediates, Compound I or in some cases peroxo-ferric complexes. In an ideal system (as in CYP101A1 with camphor) there is almost no peroxide production in this pathway, and all dioxygen consumed in the process of P450 catalysis is evenly distributed between organic products and water. However, other pathways, which do not involve product formation, also can be termed "oxygen activation," because ROS are released as a result of formation of superoxide and hydrogen peroxide using electrons from NAD(P)H. Taken together, all these pathways result in oxygen consumption and represent the process of dioxygen activation catalyzed by P450. Product and peroxide are produced with 1:1 stoichiometric consumption of NAD(P)H and O_2, while the oxidase unproductive pathway has 2:1 stoichiometry of NAD(P)H/O_2.

In order to start the activation of atmospheric dioxygen, the heme iron must be reduced to the Fe^{2+} state, to enable oxygen binding and formation of the oxy-complex of P450. Reduction is performed by electron transfer from a protein redox partner. The rate of reduction **[2] → [3]** (Fig. 3.1) in most cases strongly depends on the presence of a substrate and on the ability of this substrate to shift the spin state of ferric cytochrome P450 from low-spin (S = 1/2) to high-spin (S = 5/2). Therefore, substrates and their analogs significantly facilitate the NAD(P)H consumption and concomitantly the first step of oxygen consumption **[3] → [4]**. In case of fast autoxidation **[4] → [2]** the efficiency of the productive pathway is not high, so the overall effect of the presence of substrate may be predominantly acceleration of superoxide production. This is "the bad side" of the same coin, which is suggested to be especially important for functioning of xenobiotic metabolizing cytochromes P450 in liver, because it may cause oxidative damage by ROS production in the presence of "poor" substrates or substrate analogs that are metabolized with high uncoupling. The effect of ROS production is more pronounced if both the absolute rates of the first electron transfer and autoxidation are high, and the efficiency of the productive pathway is determined by the ratio of the rates of the second electron transfer and uncoupling.

The same logic holds for the second uncoupling branching point between protonation of Compound 0 and O–O bond heterolysis to give Compound I **[5] → [6]**, versus dissociation of peroxide **[5] → [2]**. Again, the higher these rates are, the faster the overall O_2 consumption and peroxide production if the enzyme does not provide timely delivery of protons to the distal oxygen of the peroxo-ferric complex. Protonation pathways are formed by side-chains of functionally important residues in the active site, which also help to stabilize several water molecules strategically positioned to form the hydrogen-bonded network essential for proton transfer towards the (hydro)peroxo-anion coordinated to the heme iron. The configuration and continuity of this proton-delivery network, and hence the rate and efficiency of protonation, strongly depend on the structure of substrate and it's positioning and dynamics in the vicinity of the heme. Even minor variations in the substrate structure can significantly perturb the optimal protonation network and result in highly uncoupled oxygen consumption with high absolute rates. The same is true for mutations of critically important residues, such

as T252A in CYP101A1. On the other hand, the D251N mutant in CYP101A1 highlights the role of the absolute rate of the first proton delivery in determining the overall absolute rate of catalysis with no loss of coupling.

This update, in defining the state of our understanding of P450 "oxygen activation," has encompassed many aspects of the catalytic wheel. On the nature of the species responsible for the critical transformative event of substrate into product, it is clear that there can be more than one oxidant operating. For functionalization of unactivated carbon centers, the mechanism most certainly involves radical chemistry initiated by the iron-oxo, Compound I, intermediate. This state is generated from the dioxygen bound ferrous heme by the input of a second electron and two protons that result in the cleavage of the O–O bond of atmospheric dioxygen. However, the precursor peroxo state can also be reactive in some special cases. The transformation of the initial reactants, O_2, and substrate with redox input is dependent on all the steps in the reaction cycle—from substrate binding through product release. The overall efficiency of catalysis is dependent on the protein's ability to control the critical electron and proton input and the position of the substrate near the heme active site. With $>10^4$ isozymes of P450 present throughout living organisms, this enzyme superfamily has clearly learned how to control the utilization of atmospheric oxygen and the "hot" oxidants generated upon its reduction.

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Substrate Oxidation by Cytochrome P450 Enzymes

4

Paul R. Ortiz de Montellano

4.1 Introduction

In most enzymes, the catalytic machinery is engaged throughout the process of transforming a substrate into its product, but in cytochrome P450 enzymes the catalytic machinery largely focuses on the conversion of molecular oxygen into a highly reactive oxidizing species. The subsequent oxidation of the substrate by this oxidizing species requires little or no additional catalytic assistance by the protein and is largely determined by the inherent reactivity of the oxidizing species, constraints imposed on the oxidation by steric effects, the binding orientations and mobility of the substrate within the active site, and the extent to which the various orientations of the substrate are populated. This chapter summarizes the outcome of the reactions of the oxygenating species with the different classes of functionalities and substructures in substrates.

The traditional catalytic cycle of cytochrome P450 is initiated by the binding of a substrate to the ferric enzyme, a step that is usually, but not always, accompanied by displacement of a distal water ligand from the heme iron atom (Fig. 4.1a->c). Electron transfer to the iron by an electron donor partner (Chap. 2) reduces the iron to the ferrous state (Fig. 4.1d), enabling the binding of molecular oxygen to give a ferrous dioxygen complex (Fig. 4.1e). A second electron transfer reduces this intermediate to the equivalent of a complex of the ferric iron with the hydrogen peroxide dianion (Fig. 4.1f). Protonation of the terminal oxygen in this complex produces a ferric hydroperoxy complex (Fig. 4.1g) that rapidly undergoes proton-assisted heterolytic oxygen–oxygen bond scission, generating a ferryl coupled with a porphyrin radical cation (Fig. 4.1h). In the final step, substrate oxidation by this reactive species gives the oxidized product (Fig. 4.1i) and, after product dissociation, regenerates the ferric state of the enzyme (Fig. 4.1b).

It is widely accepted that the oxidizing species responsible for most P450-catalyzed oxidations is the ferryl/porphyrin radical cation complex (Fig. 4.1h). However, the ferric hydroperoxy anion (Fig. 4.1f) can react as a nucleophile with a few electrophilic moieties, particularly carbonyl groups, usually resulting in products in which a carbon–carbon bond has been broken. A third potential oxidizing species extensively investigated in the past decade is the ferric hydroperoxide (Fig. 4.1g) that results from protonation of the ferric hydroperoxy anion. However, the current evidence suggests that this electrophilic agent is not the oxidizing species in most P450 reactions, but possibly has a limited role in the oxidation of heteroatoms and double bonds. The properties of the ferryl/porphyrin radical cation, a species analogous to that of compound I of the peroxidases, thus determine the outcome of most cytochrome P450-catalyzed oxidations.

P. R. Ortiz de Montellano (✉)
Department of Pharmaceutical Chemistry, University of California, 600 16th Street, N572D, San Francisco, CA 94158–2517, USA
e-mail: ortiz@cgl.ucsf.edu

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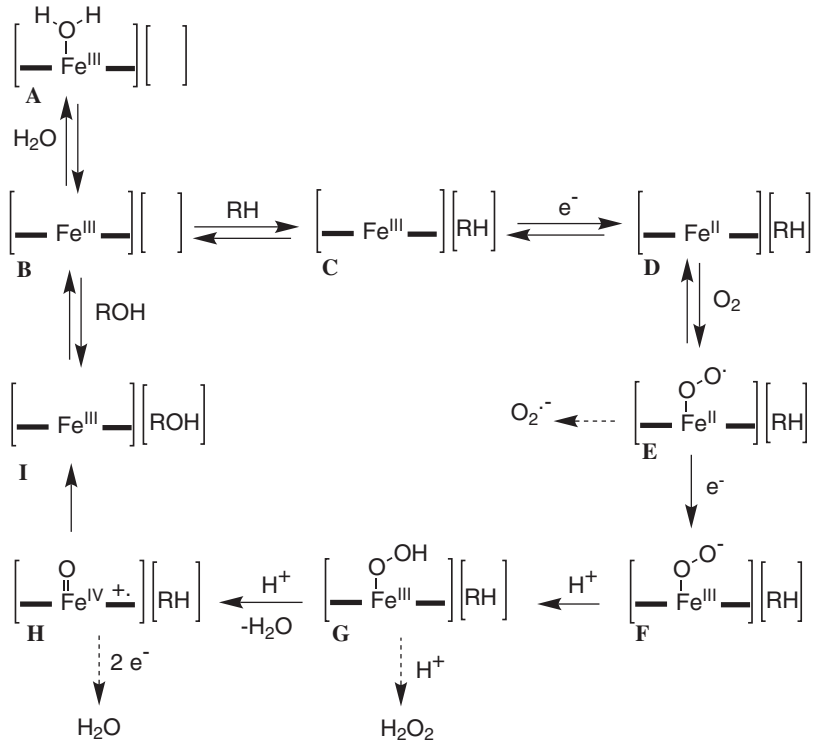

Fig. 4.1 Schematic representation of the cytochrome P450 catalytic cycle. The [Fe^{III}] represents the ferric heme of the enzyme and RH a substrate molecule. The brackets stand for the heme and substrate-binding sites. The changes occurring at the heme iron and porphyrin framework during the catalytic cycle are indicated. The electrons (e^-) required for the catalytic cycle are provided by electron donor proteins such as cytochrome P450 reductase. Three sites for uncoupling are shown that produce, respectively, O_2^-, H_2O_2, or H_2O. In this chapter, the compound I oxidizing species (*H*) is also often represented as [P^+ Fe(IV)=O], where P stands for the porphyrin framework of the heme prosthetic group. The one-electron reduced species equivalent to compound II is then represented as [PFe(IV)–OH]

The proximal ligand to the iron, which in cytochrome P450 enzymes is invariably a cysteine thiolate anion, plays a major role in determining the intrinsic reactivity of the ferryl/porphyrin radical cation. Its importance is emphasized by the fact that site-specific replacement of the cysteine ligand by a histidine [1–4], serine [5, 6], or methionine [7] yields catalytically inactive proteins. The single exception is substitution of the cysteine by selenocysteine, an amino acid that replaces the thiolate ligand by an even more electron-donating selenolate anion [8, 9]. Recent work has shown that the iron-bound oxygen in compound II of cytochrome P450 enzymes that corresponds to the state after one-electron reduction of compound I is basic, with a pK_a of ~12 for the Fe^{IV}–OH [10]. A comparison of the energy required to reduce compound I by intramolecular electron transfer from a nearby tyrosine with the energy required for hydrogen abstraction from a C–H bond in a substrate hydroxylation reaction indicates that the high basicity of the compound II Fe^{IV}–OH plays a critical role in making hydrogen abstraction competitive. The basicity of the Fe^{IV}–OH intermediate is due to electron donation from the thiolate iron ligand [11], as imidazole-ligated compound II species such as those found in peroxidases have pK_a values of ~3–6 [12]. The interaction of the thiolate with the iron can be modulated by factors such as the extent to which the thiolate is hydrogen-bonded to adjacent hydrogen bond donors [13–16]. Con-

formational differences in the heme, differences in the electrostatic properties of the heme site, and other subtle factors may further modulate ferryl reactivity, but the available evidence suggests that the ferryl properties are similar across the cytochrome P450 family of enzymes [17, 18].

The cytochrome P450 oxidation stoichiometry (Fig. 4.1) requires two electrons from NAD(P)H and one molecule of oxygen to insert one oxygen atom into a substrate. To the extent that the ratio of NAD(P)H (or oxygen) consumed to product formed is greater than one, the enzyme is said to be uncoupled. Uncoupling can occur by dissociation of superoxide from the ferrous dioxy complex (Fig. 4.1e), dissociation of H_2O_2 from the ferric hydroperoxide complex (Fig. 4.1g), or two-electron reduction of the compound I ferryl/porphyrin radical cation to a molecule of water before it can react with the substrate (Fig. 4.1h). Factors that favor uncoupling include uncontrolled access of water to the active site, population of states that place the site of reaction at unproductive distances or orientations relative to the ferryl oxygen, the absence of sufficiently reactive sites on the substrate, the efficiency of electron delivery to the heme center, and protein–protein interactions [19–24]. Uncoupling decreases the efficiency of oxidation reactions catalyzed by P450 enzymes and may contribute to the generation of deleterious reactive oxygen species (ROS). The variability of uncoupling is illustrated by the nearly quantitative coupling observed in oxidation of the natural substrate camphor by $P450_{cam}$ (CYP101) and the 95% uncoupling that is observed in the oxidation of styrene by the same enzyme [25].

4.2 Hydrocarbon Hydroxylation

The mechanism of cytochrome P450-catalyzed hydrocarbon hydroxylation, first proposed in 1978 [26], postulates abstraction of a hydrogen with its electron from a C–H bond by the compound I ferryl species, producing a substrate carbon radical and a compound II-like one-electron reduced [Fe^{IV}–OH] P450 intermediate, which can also be formally written as a complex of ferric iron with a hydroxyl radical. In the second step of this mechanism, the hydroxyl radical combines with the substrate carbon radical to produce a hydroxylated product, concomitantly regenerating the ferric enzyme (Fig. 4.2). The observation of high intrinsic isotope effects in hydrocarbon oxidations, which implies a linear rather than bent O–H–C geometry in the transition state, provides support for this mechanism. For example, the intrinsic kinetic deuterium isotope effect for rabbit CYP2B4-catalyzed 2-hydroxylation of norbornane is $k_H/k_D = 11.5$ [26] and that for the CYP3A4-catalyzed 6β-hydroxylation of testosterone is $k_H/k_D = 15$ [27]. Most intrinsic isotope effects have been determined by intramolecular competition between equivalent deuterated and undeuterated sites on the same molecule, or by more complex methods because, except for occasional instances, e.g., [28], C–H bond breaking is not the rate-determining step in the overall hydroxylation sequence. However, even isotope effects determined by intramolecular competition between equivalent sites are subject to masking due to low rates of equilibration of substrate orientations within the active site. The recent development of methods to generate high concentrations of the cytochrome P450 compound I species and

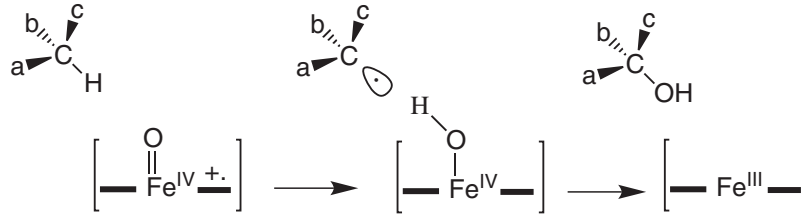

Fig. 4.2 Carbon hydroxylation generally proceeds with retention of configuration via a hydrogen abstraction, oxygen rebound mechanism. The substituents on the carbon are represented by the letters *a, b*, and *c*

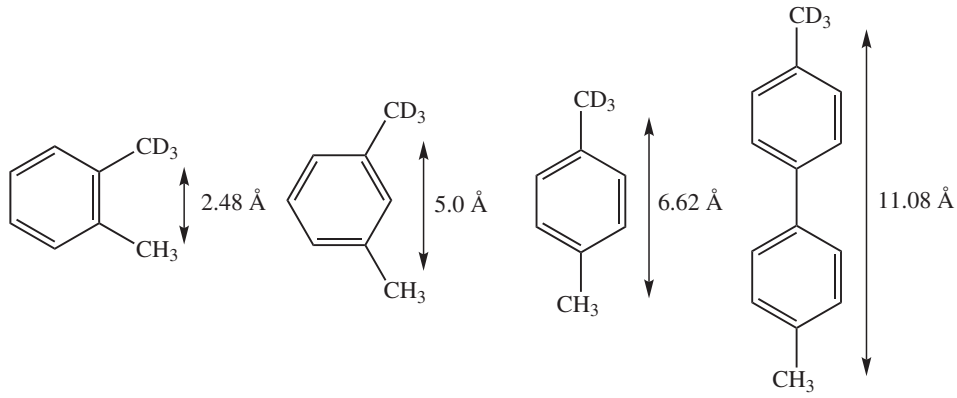

Fig. 4.3 Restricted mobility within the P450 active site can result in masking of intrinsic isotope effects, as illustrated by the finding that the isotope effect reflected by the ratio of CH_3 to CD_3 hydroxylation in the above molecules depends on the distance between the two methyl groups

to directly measure its reaction with substrates by stopped-flow methods directly confirms the generally large values obtained in earlier studies. Thus, the hydroxylation of deuterated and undeuterated hexanoic acids by CYP119 compound I reveals an isotope effect of $k_H/k_D \geq 12.5$, although this value is masked and decreases drastically as the fatty acid chain length is increased [29]. Kinetic isotope effect studies of the oxidation of fatty acids by the heme–thiolate peroxygenase from *Agrocybe aegerita,* a P450-like protein for which formation of the compound I ferryl species has also been established [30], gives an observed intramolecular isotope effect of 16 for the formation of 2-hexanol and 9 for the formation of 3-hexanol from 1,1,1,2,2,3,3-D_7 hexane [31]. In a different approach, the noncompetitive oxidation of lauric acid versus perdeuterated lauric acid by CYP105D5 gave rise to isotope effects in the range of 7–12 after correction for secondary isotope effects [32]. Density functional theoretical (DFT) calculations provide theoretical support for the interpretation of high isotope effects as evidence for a hydrogen abstraction mechanism [33, 34].

As already noted, intramolecular isotope effects can be masked if substrate mobility is so limited that repositioning of the competing oxidation sites cannot be achieved at rates sufficiently faster than the rate of the hydrogen abstraction step. This is illustrated by the P450 oxidation of a deuterated versus undeuterated methyl in *o*-, *m*-, and *p*-xylenes and 4,4'-dimethylbiphenyl (Fig. 4.3, Table 4.1) [35–37]. A much smaller intramolecular isotope effect is observed for 4,4-dimethylbiphenyl, in which the two methyl groups are separated by 11.08 Å, than for the *ortho*-, *meta*-, and *para*-xylenes, in which the methyls are separated by 2.48, 5.0, and 6.62 Å, respectively. A similar trend is seen in the isotope effects for the *ortho*-, *meta*-, and *para*-xylenes with CYP2E1, CYP2A6, and CYP101, the iso-

Table 4.1 Intramolecular isotope effects in the hydroxylation of *o*-, *m*-, and *p*-xylenes and 4,4'-dimethylbiphenyl in which one of the two methyl groups is trideuterated by four cytochrome P450 enzymes

	Xylene			4,4'-dimethylbiphenyl	Reference
	Ortho k_H/k_D	*Meta* k_H/k_D	*Para* k_H/k_D	k_H/k_D	
CYP2B1	6.66	nd	7.73	2.09	[35]
CYP2E1	9.03	6.65	6.04	2.28	[36]
CYP2A6	11.46	7.21	5.53	1.07	[36]
CYP101	10.6	nd	7.4	2.7	[37]

tope effect decreasing as the distance between the methyl groups increases. Only in the case of CYP2B1 are the isotope effects for the *ortho*- and *para*-xylenes approximately similar, suggesting that in this enzyme these two substrates have comparable mobility and their isotope effects are not significantly masked, although it was not possible to exclude the possibility that two substrate molecules were bound simultaneously in the active site. Indeed, detailed studies of the oxidation of these molecules by CYP2A6 has provided evidence for allosteric effects due to simultaneous binding of two molecules [38]. A molecular dynamics study indicated that the mobility of the compounds in the CYP101 active site decreased in the order *ortho*-xylene > *para*-xylene > 4,4'-dimethylbiphenyl, in accord with the interpretation that isotope effects were increasingly masked as repositioning of the methyls close to the ferryl oxidizing species became increasingly difficult.

Independent evidence for a hydrogen abstraction-rebound mechanism of hydroxylation is provided by the observation of stereochemical scrambling in selected cytochrome P450-catalyzed hydroxylations. The original postulate of the radical rebound mechanism was based on the finding that the hydroxylation of *exo,exo,exo,exo*-2,3,5,6-tetradeuterated norbornane yielded *exo*- and *endo*-2-norborneol in which 25% of the *exo*-2-norborneol retained four, rather than three, deuterium atoms and 9% of the *endo*-2-norborneol retained three, rather than four, deuteriums [26]. The CYP101-catalyzed 5-*exo*-hydroxylation of 5-*exo*- and 5-*endo*-deuterated camphor by CYP101 proceeds by removal of either the 5-*exo*- or 5-*endo*-hydrogen, but with exclusive delivery of the hydroxyl to the 5-*exo* position [39]. In a related system, the P450 oxidation of a derivative of camphor by the fungus *Beauveria sulfurescens* similarly resulted in loss of a hydrogen from either the 5-*exo* or 5-*endo* position, but exclusively yielded the 5-*exo*-hydroxylated metabolite [40]. The hydroxylation of phenylethane, a very different substrate than norborneol or camphor, with a stereospecifically placed deuterium at the benzylic carbon resulted in the formation of 1-phenylethanol in which 23–40% of the alcohol had deuterium in the opposite configuration to that which it had in the substrate [41]. These results require the intervention of a discrete, presumably radical, intermediate that allows inversion of the carbon stereochemistry before the hydroxyl is attached to the carbon. Despite the intervention of a radical intermediate, most P450-catalyzed hydroxylations proceed without loss of stereochemistry, as first illustrated by the retention of stereochemistry reported for the 7α-hydroxylation of cholesterol [42] and 11α-hydroxylation of pregnane-3,20-dione [43], and subsequently by the stereospecific hydroxylation of a variety of substrates, including geraniol [44], octane [45], and testosterone [46] (Fig. 4.4).

In addition to the observation of loss of stereochemistry, hydroxylations adjacent to a double bond sometimes proceed by hydrogen abstraction from the adjacent carbon, but hydroxyl attachment to the carbon at the opposite end of the resulting allylic radical. These allylic rearrangements were first observed with 3,3,6,6-tetradeuterated cyclohexene [47], methylenecyclohexane [47], β-pinene [47], 3,4,5,6-tetrachlorocyclohexene [48], and linoleic acid (Fig. 4.5) [49]. Analogous rearrangements have been reported in the hydroxylation of more complicated molecules, including the taxol precursor taxa-4(5),11(12)-diene [50], the drug exemestane [51], and a precursor of lovastatin [52] (Fig. 4.6). An even more complicated reaction involving double-bond rearrangement with simultaneous topomerization has been described for the oxidation of pulegone [53].

Radical clock probes have been used to examine the mechanism of cytochrome P450-catalyzed hydroxylations. Radical clocks refer to substrates that, if converted to free radical intermediates, undergo a free-radical rearrangement at a rate (k_r) that can be independently measured (Fig. 4.7). If the rearranged and unrearranged radicals give different products, the rate at which the radical is trapped (k_t) can be estimated from the ratio of the two products and the known radical rearrangement rate. This assumes, of course, that the rearrangement rate is not altered when it occurs within the P450 active site. The most common radical clocks are based on attachment

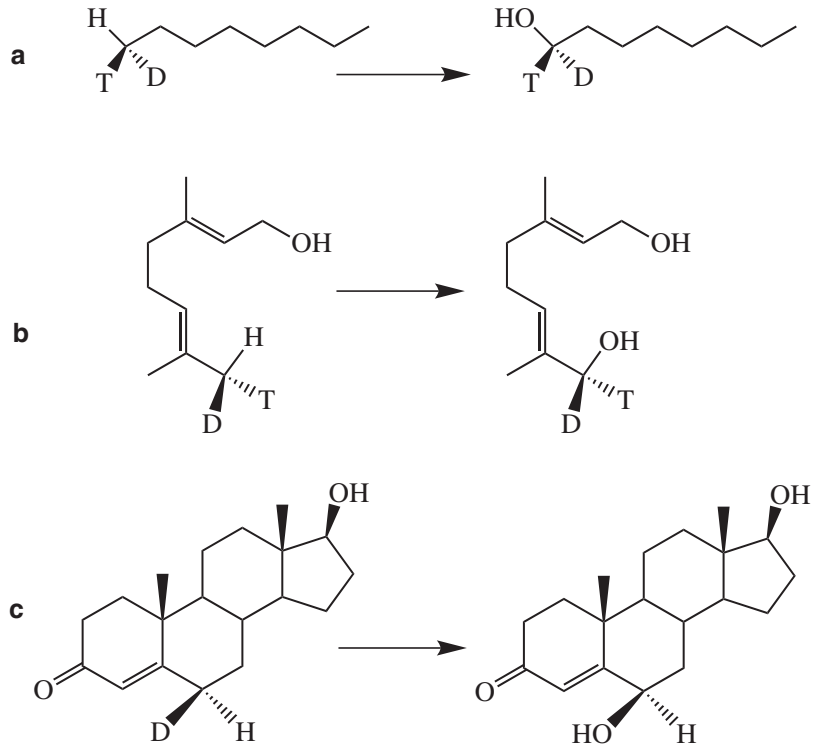

Fig. 4.4 Retention of stereochemistry in cytochrome P450-catalyzed carbon hydroxylations has been explicitly demonstrated with a variety of substrates, including octane (**a**), geraniol (**b**), and testosterone (**c**)

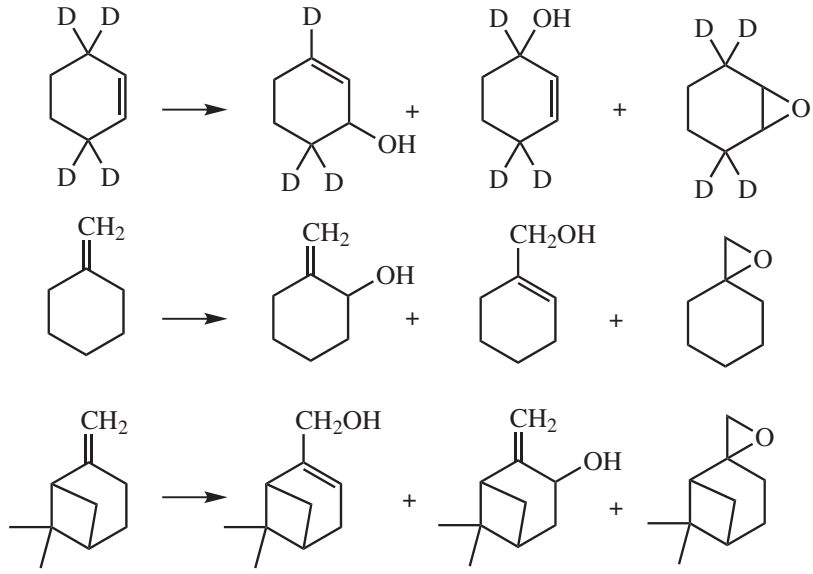

Fig. 4.5 Examples of hydroxylations in which the hydroxyl group is introduced, in part, at a position allylic to that at which the initial hydrogen abstraction occurs. A competing epoxidation may also occur in these reactions

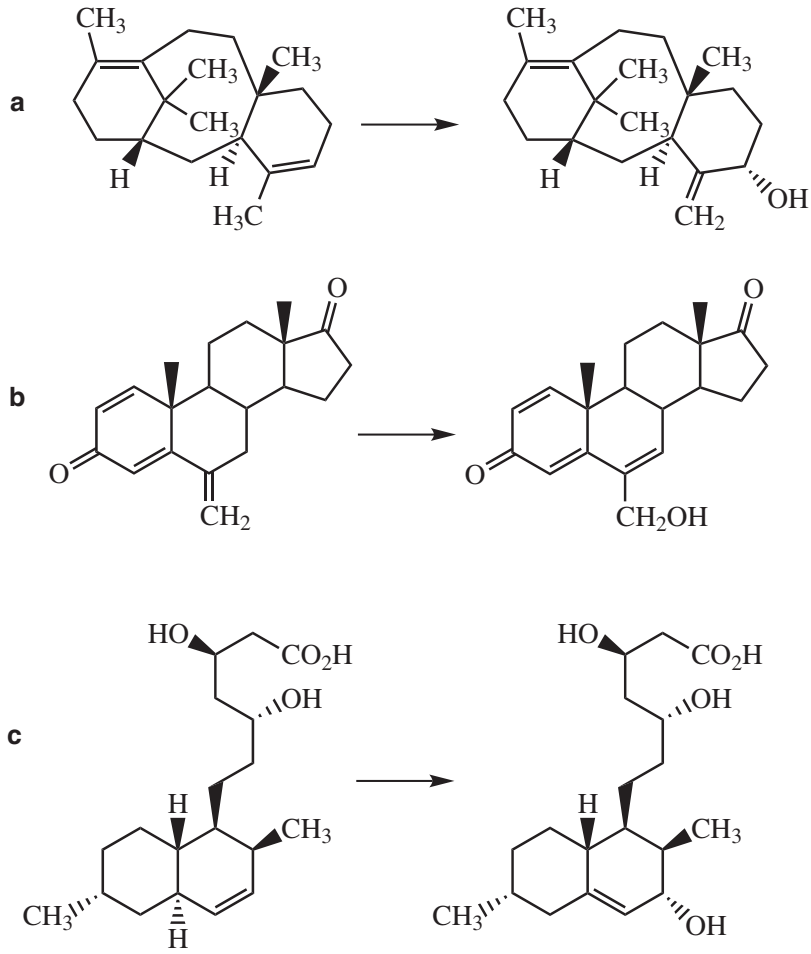

Fig. 4.6 An allylic shift of the double bond occurs in the hydroxylations of taxa-4(5),11(12)-diene (**a**), exemestane (**b**), and lovastatin (**c**)

of a strained cyclopropyl ring to the carbon at which the radical is generated. The radical rearrangement of this substructure yields a homoallylic radical. Substituents can be added to the core cyclopropylmethylene element to modulate the intrinsic rate of the radical rearrangement. The cytochrome P450 studies have shown that the radical rearrangement must occur at a rate $k_r > 10^8$ s^{-1} to compete detectably with recombination with the ferryl oxygen and thus to be useful in investigating cytochrome P450 reactions, e.g., [54, 55]. Subsequent to the introduction of radical clock probes into cytochrome P450 research, a range of radical clocks of increasing sophistication [56–63], many of which are shown in Fig. 4.7, were used to probe for the existence of radical intermediates in cytochrome P450 hydrocarbon hydroxylations. The recombination rates k_r obtained with the various P450 enzymes and radical clock probes range from approximately 2×10^{10} to 1×10^{13} s^{-1}, with most values in the 10^{10}–10^{11} s^{-1} range [62]. Recombination rates in the order of 10^{10}–10^{11} s^{-1} were also found with α- and β-thujone probes in which two separate radical "clocks," opening of the cyclopropyl group and inversion of the methyl group, operate simultaneously (Fig. 4.8) [63, 64]. The recombination rates k_t for the thujones are derived from the ratio of the ring-opened to intact-ring cyclopropylmethylene radical metabolites. Inde-

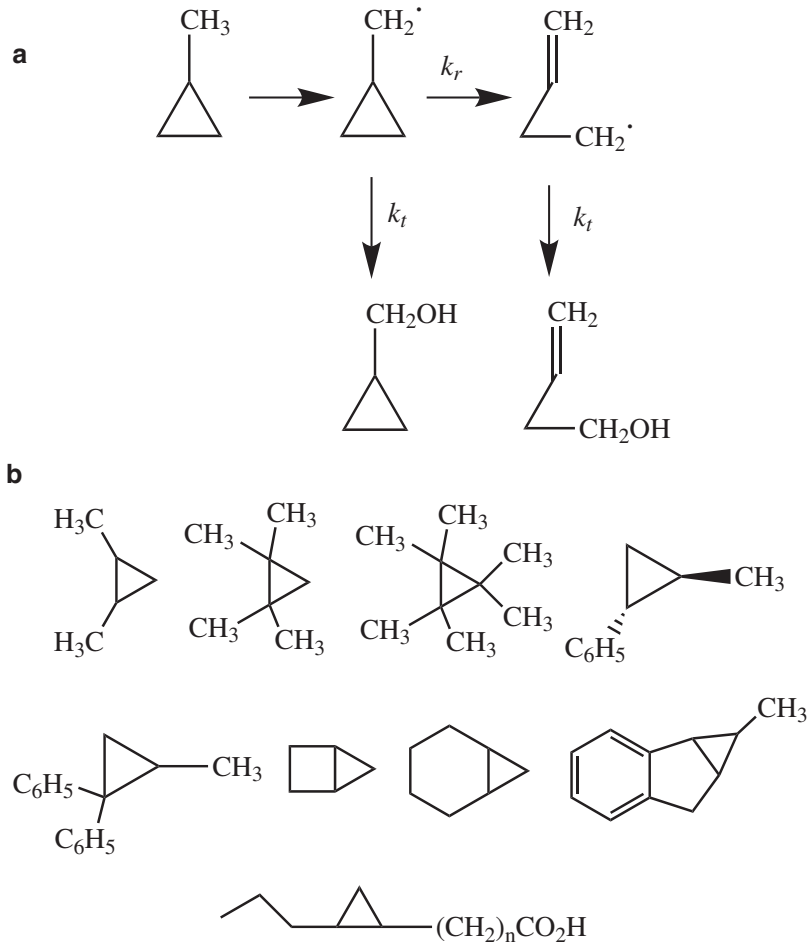

Fig. 4.7 The radical clock principle (**a**) and examples of radical clocks that have been utilized in cytochrome P450 studies (**b**)

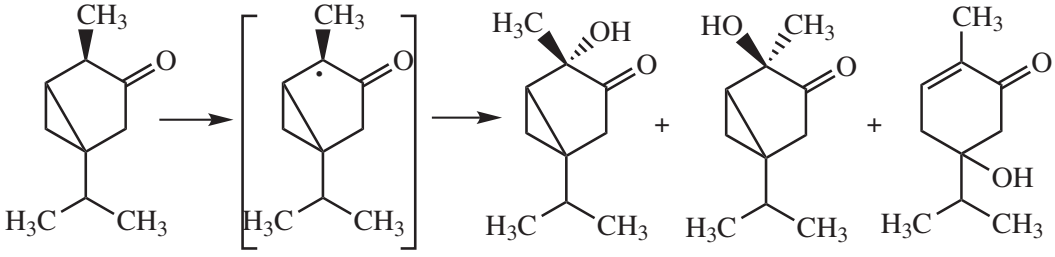

Fig. 4.8 α- and β-Thujone (β-thujone shown) function as dual radical clocks in which cyclopropyl ring opening serves as one clock and inversion of the methyl group stereochemistry as a second clock

pendent, but not timed, evidence for the radical intermediate is provided by the concomitantly observed loss of methyl stereochemistry. The collective results provide strong support for a radical mechanism, although the determination with some radical clock substrates of k_t values in the range of 10^{12} s^{-1} and higher, which approach the rates of a bond vibration, raised the question

of whether radicals existed as discrete intermediates in at least those reactions [65–67].

A theoretical rationale for the impossibly rapid radical recombination rates calculated in some instances from the ratio of rearranged to unrearranged products was provided by Shaik and coworkers [68, 69]. This rationale rests on the computational prediction that the P450 compound I ferryl intermediate exists in two distinct electronic configurations, i.e., in two different states due to the differential combination of two electrons with unpaired spins in the *d*-orbitals of the iron and a third unpaired electron in the A_{2u} orbital of the porphyrin. One of these is a doublet-spin state and the other a quartet-spin state, and these two states behave differently in the hydroxylation of a C–H bond (Fig. 4.9). Hydrogen abstraction produces a state in which the carbon radical is weakly coordinated to the iron-bound hydroxyl group. These complexes are close in energy and can again be in a doublet- or quartet-spin state, depending on whether they derive from the original compound I doublet or quartet state. A simplistic view of the resulting alternatives is provided in Fig. 4.10. If the unpaired electron on the carbon atom has a spin opposite to that of the electron in the iron–hydroxyl orbital, recombination can occur via an essentially barrierless pathway (Fig. 4.9) that is tantamount to a concerted reaction. On the other hand, the reaction via the quartet state yields a carbon in which the electron is in the same spin state as that in the iron–hydroxyl orbital, requiring a spin inversion of one of the electrons before recombination can occur. This spin inversion barrier makes the carbon radical sufficiently long-lived that it can undergo radical rearrangements before being quenched by radical recombination with the iron-bound hydroxyl. To the extent that the reaction proceeds via the doublet (virtually concerted) state to give the unrearranged product, it will distort the ratio of rearranged to unrearranged products and will result in erroneous calculation of a faster recombination rate due to the discrete radical species produced by the quartet pathway. Although the simplistic view in Fig. 4.10 is not precise in physical and computational terms, it provides an intuitive understanding of the computational results that are more precisely stated in Fig. 4.9 [68, 69].

The ferric hydroperoxide (Fe^{III}–OOH) intermediate that is the precursor of the ferryl species (Fig. 4.1g) has been proposed to be an alternative, or even primary, oxidizing species [70–72]. A number of observations led to this postulate. Thus, mutation of Thr302 in CYP2B4 and Thr203 in CYP2E1, the conserved threonines that are thought to facilitate O–O bond cleavage in oxygen activation, differentially affected the rates of oxidation of several olefins [70]. The Thr302Ala mutation in CYP2B4 decreased styrene epoxidation, cyclohexene epoxidation and hydroxylation, and *cis*- or *trans*-2-butene epoxidation and hydroxylation, but the Thr303Ala mutation of CYP2E1 increased epoxidation of all the olefins while decreasing the hydroxylation reactions. This was interpreted as evidence for the involvement of different oxidizing species in olefin epoxidation and hydroxylation, although the opposite results for the two enzymes complicate this interpretation. The ferric hydroperoxide was similarly invoked as the oxidizing agent in the CYP2B1-catalyzed oxidation of *trans*-1-methyl-2-(4-trifluoromethyl)phenyl-cyclopropane to ring-opened products [73, 74]. Analysis of the oxidation by CYP2B4 and its Thr302Ala mutant of both this substrate and the analogue with a hydrogen replacing the trifluoromethyl substituent resulted in a greater extent of phenyl than methyl oxidation in the mutant, again suggesting the intervention of a second oxidizing species, possibly related to the ferric hydroperoxide, that favored phenyl oxidation [75]. Furthermore, the oxidation of the trifluoromethyl compound with zero to three deuterium atoms on the methyl group by compound I of CYP119 and CYP2B4 gave primary isotope effects of 9.8 and 8.9 for the two enzymes, respectively [76]. Large intermolecular isotope effects k_H/k_D of 11.2 and 9.8, respectively, were found for the two compound I species, which compares with small intermolecular isotope effects found for the normal P450-catalyzed reactions. The authors interpreted this as further evidence for the existence of a second, presumably iron-complexed peroxide, in normal P450 turnover reactions.

Fig. 4.9 The two-state reaction manifold as formulated by Shaik and coworkers [68, 69]. The ferryl radical cation of compound I (*I*) has two unpaired electrons in iron d_π orbitals and one in the a_{2u} porphyrin orbital. This electron configuration can give rise to either a quartet state ($^4A_{2u}$) if all spins are unpaired or a doublet if the spin of the electron in the a_{2u} orbital is inverted. A hydrogen atom is abstracted from the substrate in the first step of the reaction and an electron is transferred to either the iron, producing the ferrous state (as shown), or to the porphyrin, neutralizing the radical cation. A quartet or doublet state is possible for either of these potential "intermediates" (*II*), depending on the pairing of the electron of the carbon radical R· with the iron porphyrin electrons. The transition of low-spin configuration *II* to low-spin product (*III*) occurs via a virtually barrierless path, whereas high-spin *II* must traverse a significant energy barrier ($^4TS_{Reb}$) to reach high-spin *III*. Therefore, only high-spin *II* behaves as a true radical intermediate with a finite lifetime. The energy diagram that corresponds to the indicated transformations is shown above the electron spin-pairing diagrams. L is the proximal iron ligand

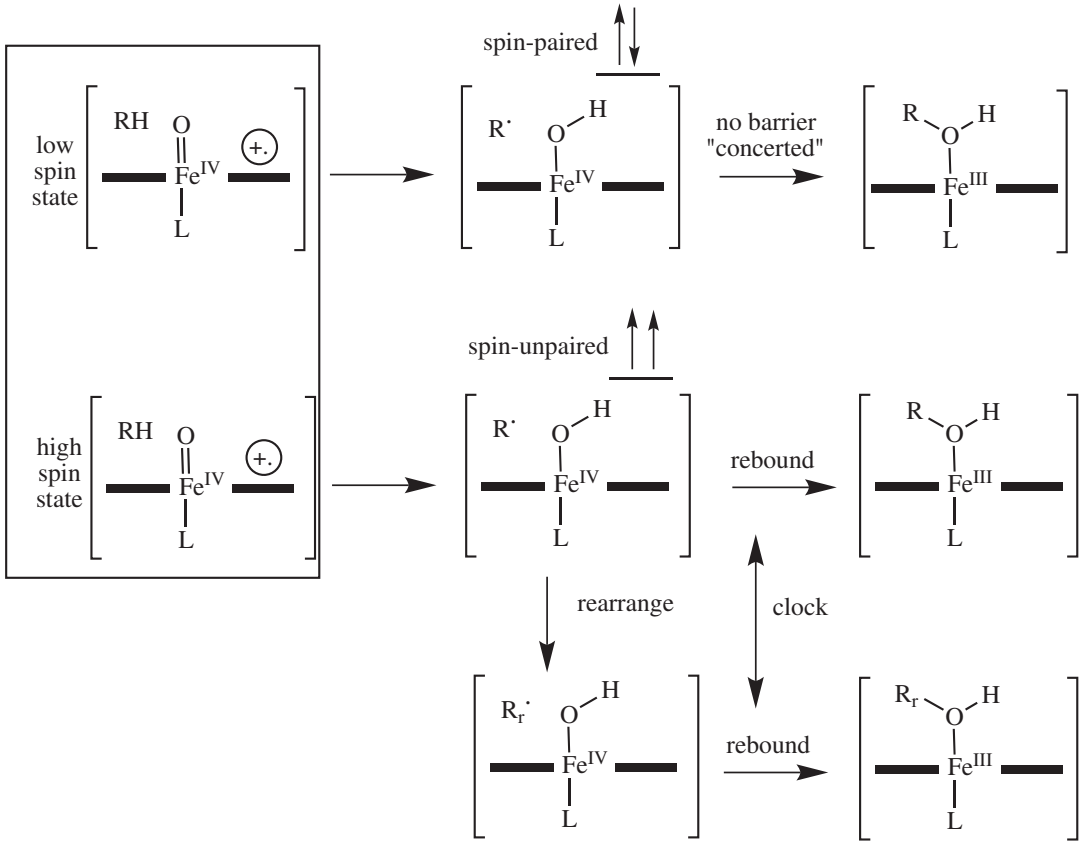

Fig. 4.10 A schematic representation of the impact of the two-state hypothesis on the timing of cytochrome P450 hydroxylation reactions by radical clocks. The rate of radical recombination is calculated from the ratio of unrearranged to rearranged products. However, as the low-spin state is virtually concerted and only gives unrearranged product, its contribution to the reaction will give rise to a deceptively high proportion of unrearranged product, some of which did not arise via the "free radical" intermediate of the high-spin state. *L* is the proximal thiolate ligand

To explore the possible role of a species other than the ferryl intermediate in substrate oxidations, Newcomb et al. generated an intermediate with an ultraviolet–visible (UV–vis) spectrum comparable to that of compound II by reaction of peroxynitrite with CYP119 [77]. Irradiation of this intermediate at 355 nm with a laser gave a low yield (~5%) of a new species with a broad Soret absorption at 400–410 nm that was attributed to compound I. However, the lifetime of this species, ~200 ms, was the same in the presence or absence of the substrate lauric acid, leading to the suggestion that the real hydroxylating species might be something else. Subsequently, Newcomb and his group obtained a better-defined "compound I" intermediate by the same method and used it to determine the intrinsic rates of oxidation of several substrates [78]. The slowest substrate studied was lauric acid, which was oxidized at a rate of 7.2×10^2 M^{-1} s^{-1}, and the fastest benzyl alcohol, which was oxidized at a rate of 2.7×10^4 M^{-1} s^{-1}. The peroxynitrite-photolysis approach was also used to generate the equivalent species in CYP2B4, which oxidized benzphetamine, a normal substrate of the enzyme, at approximately the same rate as CYP119 [79].

Identification of the species produced by reaction of a P450 enzyme with peroxynitrite followed by irradiation as a normal compound I is uncertain because the reaction of ferric CYP102 ($P450_{BM3}$) with peroxynitrite was found to yield the Fe(III)–NO complex rather than compound

II [80]. However, further investigation with CYP119 indicated that the reaction gives both a short-lived compound II species with a half-life of ~10 s at 23 °C and an Fe(III)–NO complex that was stable for hours [81]. Iron K-edge X-ray absorption spectroscopy at cryogenic temperatures indicated that the positive charge on the iron increased in going from ferric CYP119 to the Fe(III)–NO complex and finally to the compound II species, which had an iron–oxygen bond length of 1.82 Å consistent with a protonated Fe(IV)–OH structure [82–84]. In subsequent work, the Green group generated the CYP119 compound I in ~75% yield by reaction with *m*-chloroperbenzoic acid and found that its spectrum did not coincide with that reported for the species obtained by irradiation of peroxynitrite-generated compound II [82,83]. This intermediate was characterized by UV/vis, Mössbauer, and electron paramagnetic resonance (EPR) spectroscopic methods. Furthermore, compound I was shown to hydroxylate lauric acid with an apparent rate constant of 1.1×10^7 M^{-1} s^{-1} at 4 °C. Although a similar compound I had been previously detected [84], these experiments, which yielded the first biophysical characterization of compound I, also provided convincing support for the role of compound I in P450 substrate oxidations. Generation of the CYP119 compound II intermediate in a form that could be studied allowed the Green lab to show that the pK_a (Fe(IV)–OH ⇆ Fe(IV)–O^- + H^+) of the iron-bound oxygen in CYP158 is 11.9, a value to be compared with pK_a ~3–4 for hemoproteins with proximal imidazole rather than thiolate iron ligands [85]. This change in pK_a was shown thermodynamically to greatly lower the energy for hydrogen abstraction, allowing hydrogen abstraction to compete successfully with quenching of the compound I species by electron transfer from tyrosines and other oxidizable protein residues.

The role of compound I rather than its Fe(III)–OOH precursor (Fig. 4.1g) in oxidation of C–H bonds is consistent with cryogenic electron-nuclear double resonance (ENDOR) studies of the hydroxylation of camphor by CYP101 ($P450_{cam}$) [86]. Hoffman and colleagues prepared the $P450_{cam}$ ferric hydroperoxide complex at 77 K by radiolytic reduction of the camphor-bound ferrous dioxygen complex. The Fe(III)–OOH complex was shown by EPR and ENDOR experiments to be quantitatively converted at ~200 K to a complex of $P450_{cam}$ with the 5-*exo*-hydroxycamphor metabolite in which the 5-*exo*-hydroxyl group introduced by the enzyme was coordinated to the iron atom. Furthermore, ENDOR spectroscopy of the complex identified the C5–OH_{exo} and C5–H_{endo} protons, both of which disappeared when the experiment was carried out with 5,5-dideuterated camphor [87]. These results are expected from insertion of the ferryl oxygen into the C–H bond. In contrast, oxidation by the ferric hydroperoxide would have left one of the oxygens of the peroxide bound to the iron, with the other one inserted into the camphor. In order for the 5-*exo*-hydroxyl to coordinate to the iron, it would have to displace the iron-bound water molecule, an unlikely exchange reaction at 200 K.

Carbon oxidation reactions usually result in the formation of alcohol products, but in some instances they produce desaturated metabolites. Early examples are provided by the P450-catalyzed oxidative Δ^4-desaturation of valproic acid [88, 89], Δ^6-desaturation of testosterone [90], and Δ^{22}-desaturation of sterols [91, 92] (Fig. 4.11). Additional examples are provided by the desaturation of lovastatin [93], ezlopitant [94], and capsaicin [95] (Fig. 4.12). In all these examples, hydroxylation to give the normally expected alcohol product also is observed, which suggests that in these substrates desaturation diverges at some point from the normal substrate hydroxylation reaction.

Two basic mechanisms have been considered for diversion of the hydroxylation reaction to form desaturated products. In one of these, the ferryl hydrogen abstraction produces a carbon radical that is not adequately positioned for the rebound trajectory that leads to the alcohol. This imperfect alignment of the compound II iron-bound hydroxyl and the carbon radical allows transfer of an electron from the carbon radical to the iron to compete with hydroxyl transfer to the carbon radical, resulting in the formation of a carbocation. Loss of the proton adjacent to this carbocation, through either abstraction by the

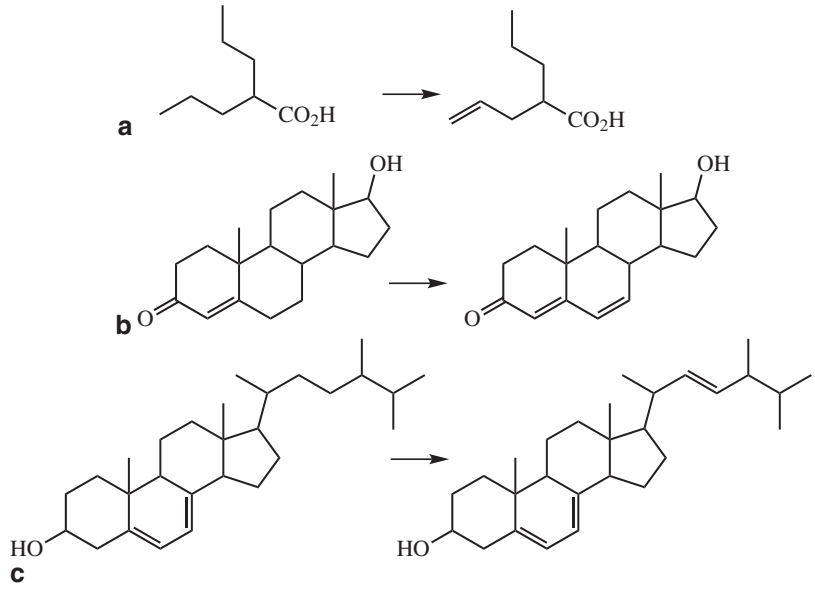

Fig. 4.11 The Δ^4-desaturation of valproic acid (**a**), Δ^6-desaturation of testosterone (**b**), and Δ^{22}-desaturation of 24-methyl-cholesterol (**c**) catalyzed by cytochrome P450 enzymes

compound II Fe(III)–OH species or an alternative proton acceptor in the active site, introduces the double bond. In the second mechanism, the compound II Fe(IV)–OH intermediate, instead of recombining with the carbon radical, abstracts a hydrogen atom from the carbon adjacent to the carbon radical, directly generating the double bond. This is illustrated in Fig. 4.13 for the desaturation of valproic acid, the best characterized of the desaturation reactions. Dissection of this reaction has shown that (a) cytochrome P450 oxidizes valproic acid to the 4- and 5-hydroxylated derivatives, but these alcohols are not converted to the desaturated product [88,96]; (b) 4-hydroxylation by phenobarbital-induced rabbit liver microsomes is subject to an isotope effect $k_H/k_D = 5.05$ when the two C4 hydrogens are replaced by deuteriums, a value comparable to $k_H/k_D = 5.58$ for desaturation of the same compound [97]; and (c) much smaller intramolecular isotope effects of $k_H/k_D = 1.62$ and 1.09 are observed for desaturation and 4-hydroxylation, respectively, when the three terminal methyl hydrogen atoms are replaced by deuteriums [97]. Further studies with CYP2B1 and CYP4B1 showed that the ratios of hydroxylation to desaturation were 37:1 and 2:1 for these two proteins, respectively, and the corresponding k_H/k_D values for desaturation of 4-dideuterated valproic acid were 3.6 and 7.6 [98]. Much smaller isotope effects were found for desaturation of 5-trideuterated valproic acid. These results show that removal of the C4 hydrogen is subject to a large isotope effect, but loss of the hydrogen at C5 is not. It is striking, given that CYP4B1 primarily (but not exclusively) catalyzes valproic acid 5-hydroxylation, that desaturation appears to arise even with this enzyme largely or exclusively via abstraction of the C4 hydrogen.

The formation of hydrocarbon cations suggested by the formation of desaturation products during P450 hydrocarbon hydroxylation reactions finds support in other experiments. Newcomb et al. synthesized the first probe that functioned competitively as both a radical clock and a cation sensor [99]. The probe (Fig. 4.14) can undergo normal hydroxylation (path a), opening of the cyclopropylmethylene radical intermediate to give the resonance-stabilized benzylic radical (path b), or, after oxidation to the cation, ring opening to place the positive charge adjacent to the stabilizing methoxy oxygen (path c). In ef-

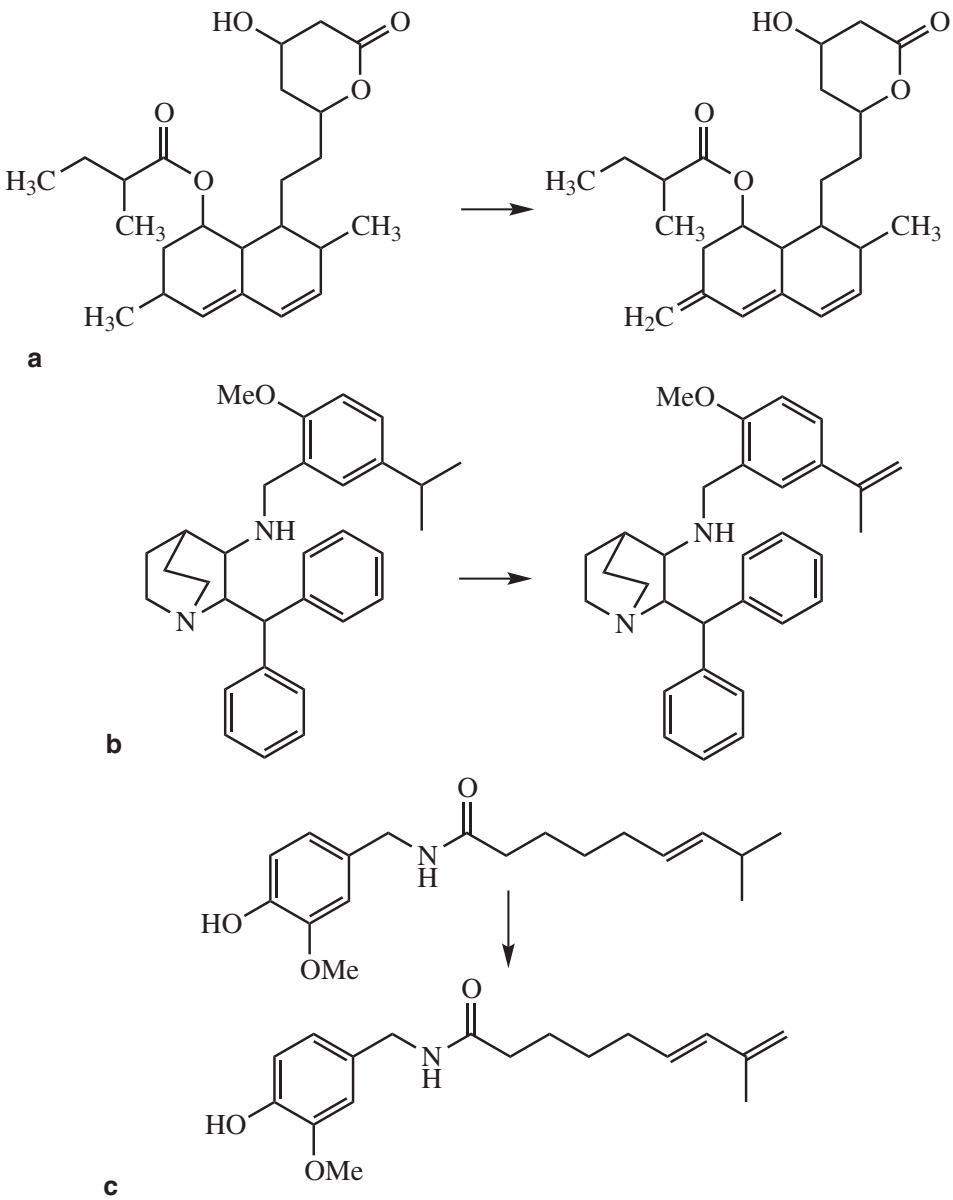

Fig. 4.12 Cytochrome P450-catalyzed desaturation of the drugs lovastatin (**a**), and ezlopitant (**b**), and of the natural product capsaicin (**c**)

fect, the cation-derived products were obtained in 2–15% yield in the oxidations of this probe by CYP2B1, CYP2B4, and CYP2E1 [99]. Other probes that undergo different radical versus cation rearrangements include α- and β-thujone [64] and an *exo*-methyl cubane derivative (Fig. 4.14) [99], all of which showed that carbocation formation occurred as a minor pathway. In contrast, fatty acids with mid-chain cyclopropyl groups gave radical but not cation rearrangement products [100].

Shaik's two-state model for hydrocarbon hydroxylation readily rationalizes the available mechanistic evidence. As already noted, the initial hydrogen abstraction can be mediated by both the low-spin (LS) and high-spin (HS) electromers of the ferryl species. However, after hydrogen abstraction, the LS species decays by a

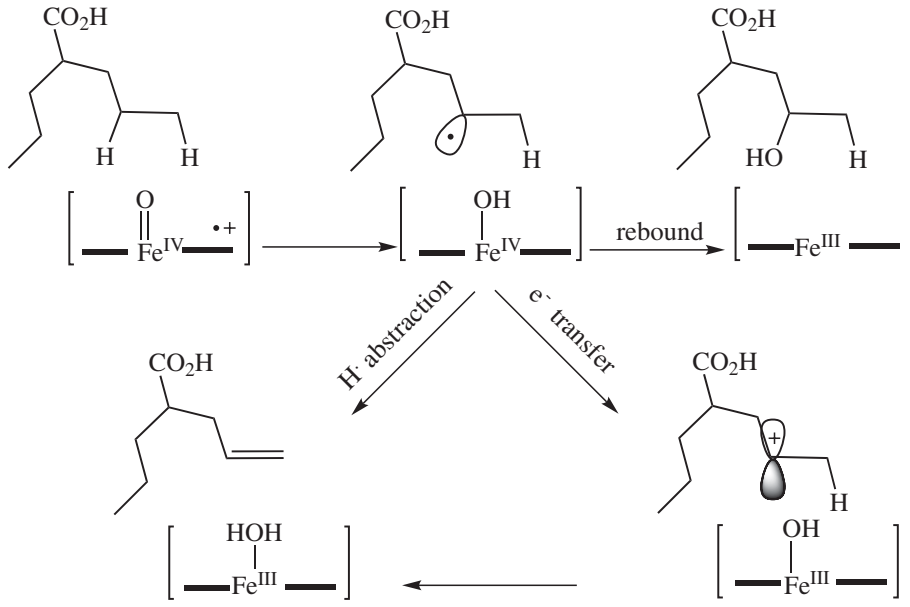

Fig. 4.13 Two limiting mechanisms for the desaturation of valproic acid are illustrated. In one, a second hydrogen is abstracted by compound II generated after initial hydrogen abstraction by compound I, whereas in the other the first hydrogen abstraction is followed by an electron transfer to the ferryl species, resulting in carbocation formation. A proton loss then completes the reaction

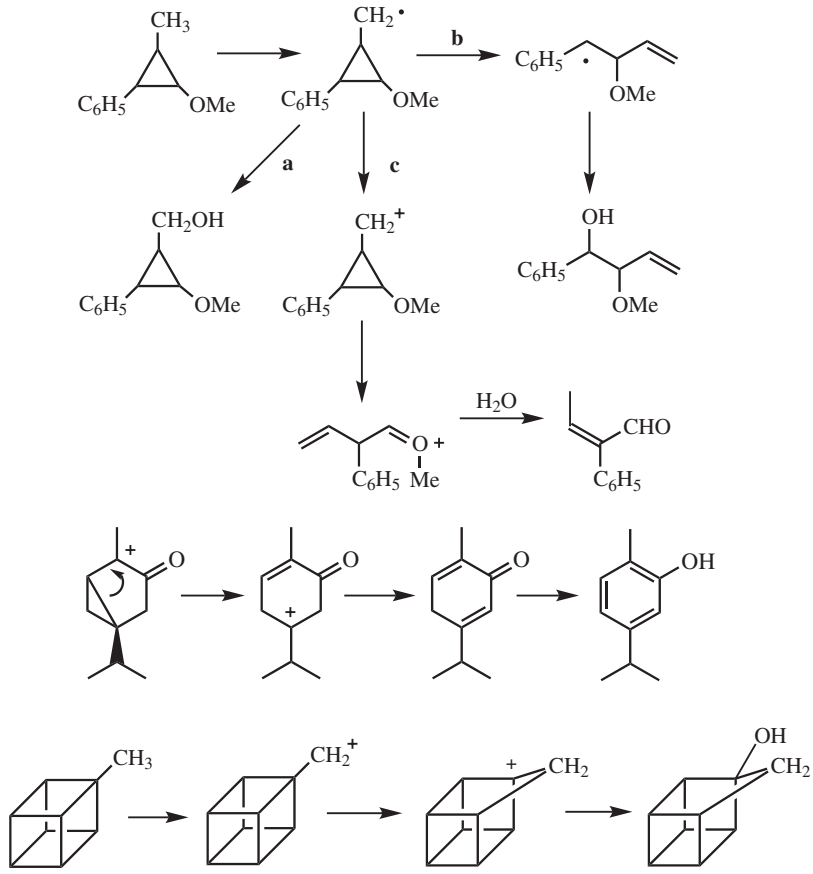

Fig. 4.14 Probes designed to test for the involvement of radical cation intermediates in cytochrome P450 catalysis

barrierless, essentially concerted, pathway to the unrearranged alcohol, whereas the equivalent HS species must traverse a significant energy barrier before recombination can occur, resulting in the formation of a true radical intermediate. Carbocations may be formed with some substrates, usually as minor intermediates, by a mechanism that presumably involves electron transfer from the radical to the compound II ferryl species.

Three factors determine the specificity of cytochrome P450-catalyzed carbon hydroxylation reactions. One is the binding affinity of the substrate for the enzyme, as defined by the dissociation constant K_d and the Michaelis turnover constant K_m. This affinity is controlled by the fit of the substrate within the enzyme active site, its lipophilicity, and whatever hydrogen bonding or other specific interactions may exist between the substrate and active residues. The second factor is the intrinsic reactivity of the individual C–H bonds in the molecule, which is directly related to their bond strength. Finally, the relative ease of oxidation at various positions in a substrate depends on the degree of mobility of the substrate in the active site and the extent to which individual C–H bonds can be placed in a proper position and orientation for hydrogen atom abstraction by the ferryl oxygen.

A major factor in determining the binding affinity of a compound for a P450 active site is its lipophilicity, the general observation being that the more lipophilic the compound is, the more tightly it is bound. This assumes, of course, that the compound is accepted into the active site of the P450 enzyme. This relationship between lipophilicity and binding affinity reflects the fact that P450 active sites are more lipophilic than the surrounding aqueous medium, so that increasing lipophilicity favors partitioning into the protein active site. The relationship between lipophilicity and affinity has been formalized by many studies showing that the K_d (often measured spectroscopically and therefore given as K_s) or K_m of a compound decreases, reflecting enhanced binding, as its lipophilicity increases, e.g., [101–103]. For example, hydroxylation of the methyl group of 4-substituted toluenes by CYP2B4 adheres to the Hansch equation:

$$\log k_{cat} = 0.53C\log P - 0.77\sigma - 0.67, \quad (4.1)$$

where $C\log P$ is a calculated lipophilicity parameter and σ is the usual Hansch electronic parameter. A second example is the good correlation that exists between the $-\log K_m$ and the octanol–water log P values of 16 diverse substrates in their catalytic turnover by CYP2B6, as given by the equation [105]:

$$-\log K_m = 0.881 \log P + 1.676. \quad (4.2)$$

In terms of the intrinsic reactivity of C–H bonds, the rate-limiting step in their cytochrome P450-catalyzed hydroxylation is abstraction of the hydrogen by the compound I ferryl species, a reaction that can be written as shown in Eq. 4.3:

$$[P^{+\cdot}Fe(IV)=O] + C{-}H \rightarrow [PFe(IV)-OH] + C^{\cdot}, \quad (4.3)$$

where $P^{+\cdot}$ stands for a porphyrin radical cation. As the changes at the porphyrin and the iron are the same for all carbon hydroxylations, the intrinsic reactivity of a C–H bond is closely related to its bond strength, which is defined as the energy required for the reaction $C{-}H \rightarrow C^{\cdot} + H^{\cdot}$. Furthermore, as the energy of $H^{\cdot}$ is the same for all the reactions, the critical factor is the stability of the carbon radical that is formed; the more stable the radical, the less energy is required to break the C–H bond and the higher its "intrinsic" reactivity in P450 hydroxylation reactions. Thus, from bond strength considerations alone (Table 4.2), one would predict that the order of hydrocarbon C–H bond oxidation would be benzyl ~ allyl > tertiary > secondary > primary. Indeed, early experiments with microsomal P450 preparations showed that the intrinsic reactivity of hydrocarbon C–H bonds increased in going from a primary to a secondary to a tertiary C–H bond (Fig. 4.15) [108]. In all the compounds shown, oxidation of a tertiary C–H bond is highly favored if one is present, and secondary C–H bonds are oxidized more readily than the primary C–H bonds of methyl groups. It is to be noted, however, that steric effects are superimposed on the intrinsic C–H bond reactivity, so that the central carbon

Table 4.2 Molecular bond dissociation energies for selected C–H bonds

Bond	Kcal mol^{-1}	Reference
C_6H_5–H	112.9	[106]
CH_3–H	105.0	[106]
CH_3CH_2–H	101.1	[106]
$(CH_3)_2CH$–H	98.6	[106]
$(CH_3)_3C$–H	96.5	[106]
$C_6H_5CH_2$–H	89.8	[106]
$CH_2=CHCH_2$–H	88.8	[106]
$HOCH_2$–H	96.1	[106]
$HSCH_2$–H	94	[106]
H_2NCH_2–H	92.2	[107]

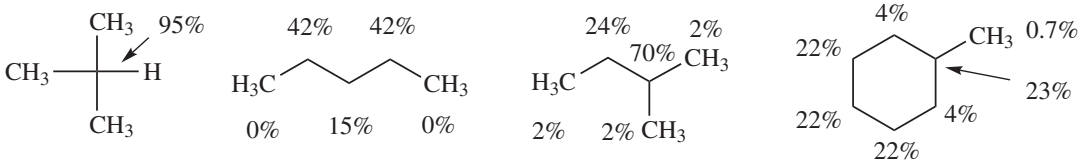

Fig. 4.15 Regiospecificity of hydrocarbon hydroxylation by liver microsomal cytochrome P450 enzymes. The percent of the total hydroxylation at each site of these small hydrocarbons is reported, with the percent for equivalent sites divided equally among them

in heptane is oxidized to a lower extent than the methylenes adjacent to the terminal·carbons, and the methylene groups flanking the carbon with the methyl group in methylcyclohexane are oxidized less efficiently than the other methylenes. Studies by Korzekwa and colleagues calculated the reactivity of different C–H bonds by semiempirical quantum chemical calculations using a model in which the hydrogen abstraction was mediated by a *p*-nitrosophenoxy radical [109]. Subsequent calculations using a variety of computational methods agree with the earlier conclusion that the bond strength of the C–H bond is the critical factor in determining its intrinsic reactivity, although steric effects within the substrate molecule and preferences imposed by the binding and mobility of the substrate within the specific cytochrome P450 active site can alter inherent reactivity differences [110–112]. For example, the CYP4A P450 family preferentially oxidizes the terminal methyl of fatty acid chains, whereas most P450 enzymes hydroxylate the methylene adjacent to the terminal methyl. In a chain, the terminal methyl is known as the ω-position, after the last letter of the Greek alphabet, and positions down the chain from it are known as ω-1, ω-2, etc. The ω-hydroxylation specificity of CYP4A enzymes requires specific structural constraints that override the inherent preference for oxidation of the sterically less accessible but weaker ω-1 C–H bond. A second example is provided by the CYP3A4-catalyzed hydroxylation of terfenadine that leads eventually to fexofenadine in preference to benzylic hydroxylation or oxidation of the relatively weak C–H bonds adjacent to the nitrogen (Fig. 4.16).

The compound I species of the *Agrocybe aegerita* peroxygenase, a P450-like enzyme that utilizes peroxides rather than NAD(P)H and molecular oxygen to generate its compound I intermediate, has been formed with *meta*-chloroperbenzoic acid. Its decomposition rate was slow enough that its rate of hydroxylation of various hydrocarbons could be directly measured [113]. The rates of reaction of compound I were found to be linearly correlated with the bond dissociation energies (BDE) of the C–H bonds, but they became insensitive to the BDE at values below 90 kcal mol^{-1}. A linear correlation of hydroxylation rates with the BDE was also reported for compound I of CYP119 generated by photolysis of compound II [114].

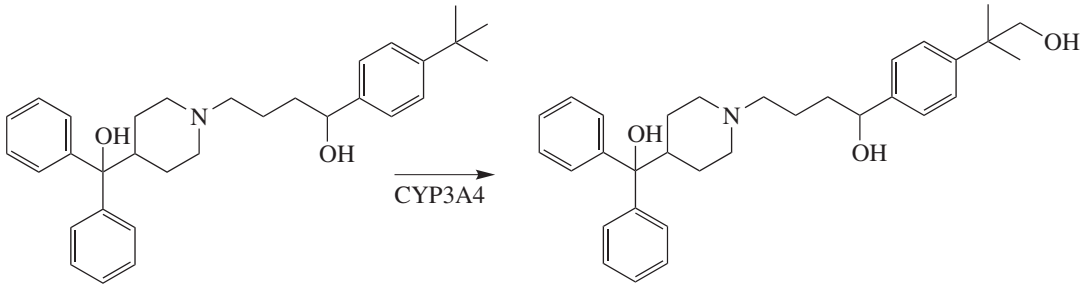

Fig. 4.16 Hydroxylation of a methyl carbon in terfenadine. Two subsequent P450-catalyzed oxidations convert the alcohol metabolite to the acid that is present in fexofenadine

4.3 Hydroxylation Adjacent to a Heteroatom

The cytochrome P450-catalyzed transformations commonly known as *O*-, *N*-, and *S*-dealkylations, as well as oxidative deamination and dehalogenation, involve the introduction of a hydroxyl group on a carbon adjacent to the heteroatom, followed by intramolecular elimination of the heteroatom with concomitant generation of a carbonyl moiety (Fig. 4.17). The carbon hydroxylation in *O*-dealkylation and oxidative dehalogenation occurs

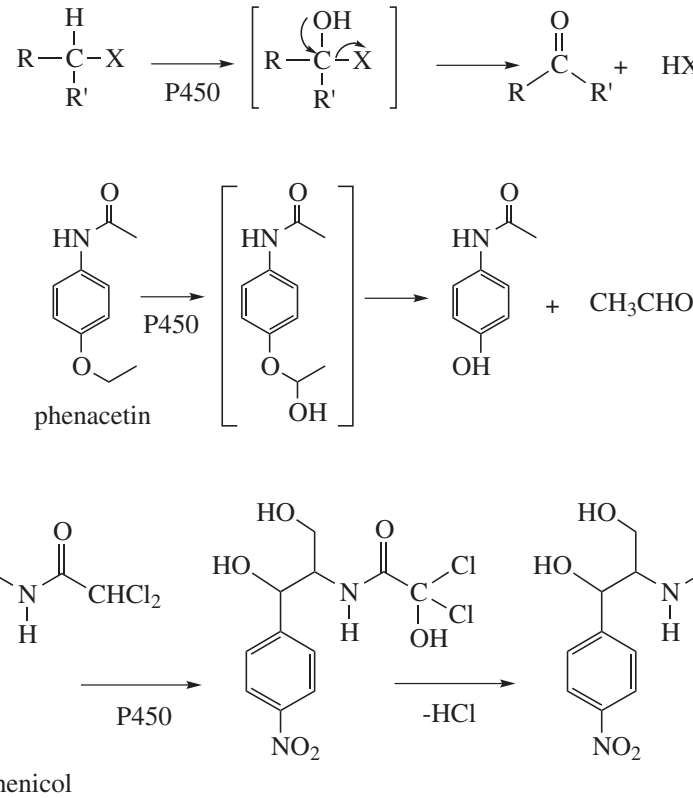

Fig. 4.17 Hydroxylation of a carbon atom with a heteroatom substituent (X) attached to it usually results in elimination of the heteroatom with formation of a carbonyl group on the hydroxylated carbon. Phenacetin *O*-dealkylation and chloramphenicol oxidative dehalogenation are two examples of this general reaction

by the same mechanism as hydrocarbon hydroxylation, with the compound I ferryl abstracting a hydrogen to generate a carbon radical that collapses with the iron-bound hydroxyl "radical" to produce the alcohol. However, hydroxylation of an oxygen-substituted carbon is facilitated by the fact that the BDE is lower for a C–H adjacent to an oxygen than adjacent to a carbon (Table 4.2). In accord with this mechanism, the intramolecular isotope effects for *O*-dealkylation are high, with $k_H/k_D \sim 13$ for *O*-deethylation of deuterated 7-ethoxycoumarin [115] and ~10 for *O*-demethylation of trideuteromethyl 4-nitroanisole [116]. These reactions differ from simple hydrocarbon hydroxylations in that the newly introduced hydroxyl group rapidly extrudes the ether oxygen (or halogen atom), as illustrated in Fig. 4.17 for phenacetin [117] and chloramphenicol [118], classic examples of *O*-dealkylation and oxidative dehalogenation.

Hydroxylation adjacent to a nitrogen is more complicated because the relatively low electronegativity of nitrogen enables two distinct limiting mechanisms. As in *O*-dealkylation, one of these mechanisms involves generation of a carbon radical by hydrogen abstraction followed by recombination with the iron-bound hydroxyl, resulting in hydroxylation of the carbon to which the nitrogen is attached. The second mechanism yields the same hydroxylated metabolite, but via a different reaction sequence. This alternative route is initiated by one-electron transfer from the nitrogen to compound I, producing a nitrogen radical cation. Loss of a proton from a carbon attached to the nitrogen then gives, after electron redistribution, a carbon radical that collapses with the iron-bound oxygen to form the hydroxylated product (Fig. 4.18). The first sequence is an example of a hydrogen atom transfer (HAT) mechanism and the second of a single electron transfer (SET) mechanism. By whichever mecha-

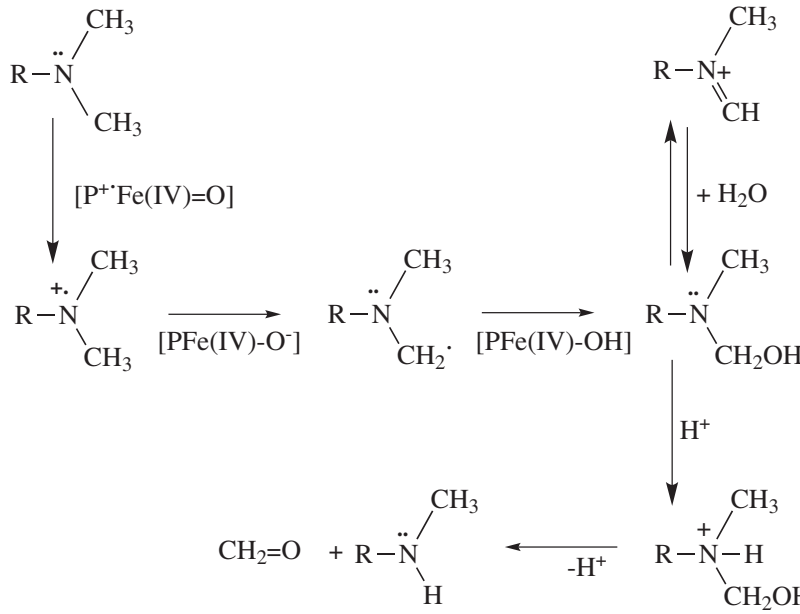

Fig. 4.18 An alternative mechanism is available for hydroxylation of a carbon adjacent to a nitrogen atom. This mechanism is initiated by electron transfer from the nitrogen to compound I, forming a nitrogen radical cation and compound II. Proton removal and recombination with the iron-bound hydroxyl of compound II then yields the hydroxylated product. The high electronegativity of the heteroatom makes this mechanism energetically inaccessible for reactions where it is an oxygen or halogen. Furthermore, the lower electronegativity of nitrogen enables it to competitively extrude the hydroxyl group to give an iminium metabolite, although this product is usually unstable relative to water addition to regenerate the alcohol metabolite

nism, a common hydroxylated product is formed that usually fragments by an acid-catalyzed reaction to give the dealkylated amine and a carbonyl moiety.

The relative roles of the SET and HAT mechanisms in the *N*-dealkylation of xenobiotics continue to be a matter of debate. In those instances in which the nitrogen electron pair is strongly tied up in a conjugated system, as in *N*-alkylamides, the reaction appears to proceed largely by a HAT mechanism. Support for this is provided by the observation that amide *N*-dealkylations are subject to large intramolecular isotope effects, in contrast to *N*-dealkylation reactions in which the nitrogen electron pair is less tied up by conjugation. Thus, the minimum intramolecular kinetic isotope effect for demethylation of *N*-trideuteriomethyl-*N*-methylbenzamide was independently determined to be 6.55 and 6.0 [119, 120]. This isotope effect was largely masked in intermolecular experiments, for which the V_{max} isotope effects of 0.9 and 1.23, and V_{max}/K_m isotope effects of 1.4 and 1.75, were measured [119, 120]. In contrast, the intramolecular isotope effect for electrochemical *N*-demethylation of the same substrate, a reaction that clearly proceeds via the radical cation, was 2.78 [121].

The situation is less clear for the *N*-dealkylation of substituted *N*-alkylanilines and compounds with unconjugated nitrogen atoms. The isotope effects for these *N*-dealkylation reactions are low and comparable to those for electrochemical *N*-dealkylations. For example, the intramolecular isotope effect for *N*-demethylation by CYP1B1 of six *para*-substituted *N*-methyl-*N*-trideuteromethylanilines ranged from $k_H/k_D = 1.56$ to 2.27, with the *para*-nitro compound having a higher value of 3.56 [122]. Comparably low isotope effects ($k_H/k_D = 2.3–3.3$) resulted when *para*-substituted *N*-methyl-*N*-trideuteromethylanilines were dealkylated by a model system consisting of an iron porphyrin and iodosobenzene [123]. Not surprisingly, given the electron-deficient nature of the compound I ferryl species, the rates for the oxidation of 12 *p*-substituted *N, N*-dimethylanilines by rat liver microsomes were well described by the equation $\log V_{max} = 0.41\pi - 1.02\sigma - 0.023MR + 1.72$ ($r = 0.953$), where π is the log of the partition coefficient, σ the Hammett electronic factor, and MR the molecular refractivity, a measure of steric bulk [124]. In similar experiments using a purified cytochrome P450 in which log V_{max} was plotted versus the substituent Hammett electronic factor σ, the slope was found to be −0.61 for the normal enzymatic reaction and −0.74 for the reaction supported by iodosobenzene [125]. The negative coefficients for σ in these relationships indicate that the reaction is accelerated by electron-donating substituents, in agreement with the formation of a nitrogen radical cation by an SET mechanism, but also consistent with a HAT mechanism, as it, too, would be facilitated by electron donation.

Direct evidence exists for the formation of nitrogen radical cations in the oxidation of amines by peroxidases, but the evidence for their formation in cytochrome P450-catalyzed amine oxidations is indirect. Nitrogen radical cations have not been directly observed by EPR or other spectroscopic means in the normal catalytic turnover of amines by P450 enzymes, although colored aminium radicals were observed in the oxidation of some amines by CYP2B1 supported by iodosobenzene [116]. Indirect evidence for a nitrogen radical cation is provided by the observation that the 4-alkyl group of 3,5-(*bis*)carbethoxy-2,6-dimethyl-4-alkyl-1,4-dihydropyridines is eliminated upon P450 oxidation as a radical that alkylates the P450 prosthetic heme group [126]. A spin-trapped ethyl radical has also been detected in incubations of 4-alkyl-1,4-dihydropyridines with liver microsomes, but the extent to which the spin-trapped radical arises from P450 catalysis as opposed to oxidation of the substrate by trace metals is unclear [127, 128].

As discussed earlier, radical clocks in which a cyclopropyl ring is attached to a carbon radical generated by P450-catalyzed hydrogen abstraction have been used to examine the lifetime of the radical. A similar approach can theoretically be used to probe for the formation of nitrogen radical cations in amine oxidations, as a cyclopropyl ring attached to a nitrogen radical cation also undergoes a ring-opening reaction. P450-catalyzed formation of a radical cation from cyclopropyl

amines, followed by ring opening to give an iminium carbon radical that alkylates the heme group, was postulated to explain the inactivation of P450 enzymes by such substrates [129, 130]. Correlation of the rates of P450 inactivation by a series of heteroatom-substituted cyclopropanes with their one-electron oxidation potentials provided some support for a radical cation mechanism [131]. However, measurements of the rate of ring opening of *N*-cyclopropylaniline radical cations generated electrochemically or by photoionization indicate that the cyclopropyl ring opens at a rate of 4.1×10^4 s^{-1} [132]. This very slow rate is to be compared to the ring-opening rates of $>10^8$ s^{-1} that are required for hydrocarbon radical clocks to effectively compete with the normal recombination step in hydrocarbon hydroxylation [62]. *N*-Cyclopropylanilines are therefore unlikely to be useful as reporters for the intervention of nitrogen radical cations in P450-catalyzed nitrogen oxidations.

Horseradish peroxidase (HRP), which demethylates *N,N*-dialkylanilines in the presence of H_2O_2 and O_2 [133], oxidizes *N*-cyclopropyl, *N*-methylaniline to *N*-methylaniline and a product that arises via radical ring opening of the cyclopropyl group (Fig. 4.19). In contrast, the only product identified in the microsomal P450-catalyzed oxidation of *N*-methyl, *N*-cyclopropylaniline was the hydrated form of cyclopropanone [134]. Oxidation of *N*-methyl, *N*-(1-methylcyclopropyl)aniline, in which the cyclopropyl carbon has no hydrogen, resulted in *N*-demethylation and *para*-hydroxylation, but no cyclopropyl ring-opened products. Likewise, the CYP101-catalyzed oxidation of *N*-methyl, *N*-cyclopropylaniline supported by 2,3,4,5,6-pentafluoro-*N, N*-dimethylaniline *N*-oxide, a surrogate activated oxygen donor, yielded *N*-dealkylated products without detectable opening of the cyclopropyl ring [135]. These results are consistent with a HAT mechanism in which hydroxylation occurs at the cyclopropyl carbon and provide no support for a nitrogen radical cation mechanism, although the significance of this finding is compromised by the slow rate of opening of a cyclopropyl ring attached to a nitrogen radical cation. In related work, the oxidation of *N*-(alkyl)cyclopropyl-*N*-cyclopropyl-*p*-chloroaniline by HRP and CYP2B1 was examined [136]. Oxidation of the dicyclopropyl probes by CYP2B1 and rat liver microsomes gave the metabolites in which one or the other of the cyclopropyl groups was removed, and for the isomer with the methyl and nitrogen

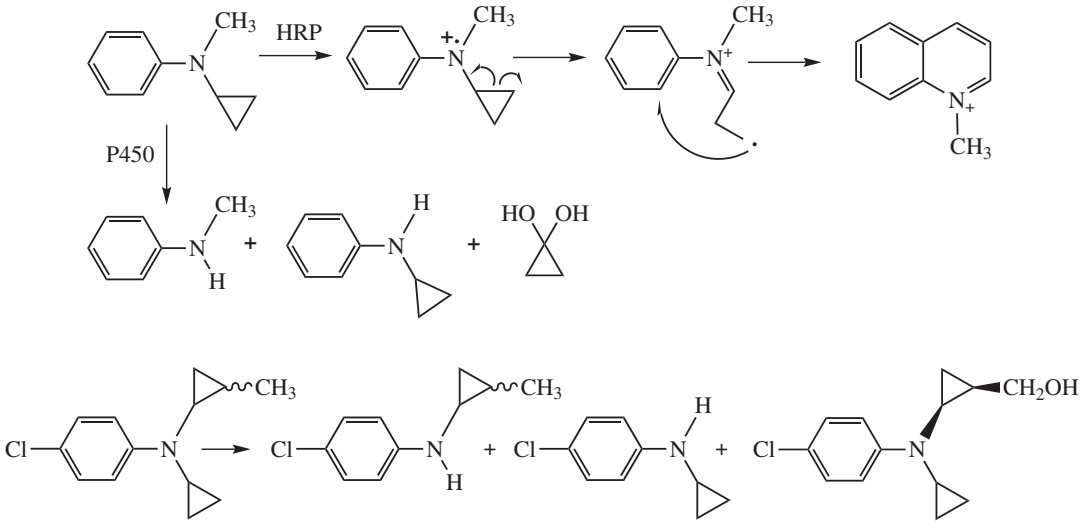

Fig. 4.19 Although a nitrogen radical cation can be detected by rearrangements of cyclopropylamine probes in reactions with horseradish peroxidase (*HRP*), similar rearrangements are not observed in the corresponding cytochrome P450-catalyzed reactions

cis to each other, also a major amount of methyl hydroxylation (Fig. 4.19). This contrasts with the HRP-catalyzed oxidation of the same substrates, which exclusively yields products from opening of the methyl-substituted cyclopropyl ring, as expected for a reaction proceeding via the nitrogen radical cation [136]. These results led to the conclusion that *N*-dealkylations proceed via a HAT rather than SET mechanism. A comparison of the electrochemical oxidations of *N*-methyl- and several *N*-cyclopropyl-4-phenyl-1,2,3,6-tetrahydropyridines, which indicated that opening of the cyclopropyl ring was highly favored, with the products observed for these compounds in P450-catalyzed oxidations suggested that nitrogen radical cations are not obligatory intermediates in *N*-dealkylation reactions [137]. Indeed, the authors proposed that these reactions also proceed via a HAT pathway.

Further information relevant to P450-catalyzed *N*-dealkylations is provided by comparison with the analogous reactions catalyzed by HRP. A correlation exists between the rates of reduction of HRP compound I and the oxidation potentials of *para*-substituted *N,N*-dimethylanilines and *N,N*-di(trideuteriomethyl)anilines [138]. Furthermore, only low isotope effects were observed in these reactions, as well as reactions catalyzed by hemoglobin and prostaglandin synthase [139, 140]. This contradicted earlier studies in which product formation rather than compound I reduction was measured, studies that suggested that *N*-demethylation of *N,N*-dimethylaniline by HRP was subject to a large isotope effect [116, 141]. As reported, product formation is a misleading index of reactivity in these reactions, as product formation involves a disproportionation reaction of the initially formed nitrogen radical cation that is subject to a large isotope effect [139]. Based on these findings, the HRP reaction was attributed to an SET mechanism rather than the earlier postulated HAT mechanism.

Computationally, Shaik and coworkers have predicted that the two electromer spin states of compound I react differentially in the *N*-dealkylation of *N,N*-dimethylaniline [142]. The calculated energies indicate that the barriers for C–H hydroxylation are low, in accord with the relatively weak C–H bond energies of the N–CH_3 hydrogen atoms. There is a calculated energy difference between the low- and high-spin states of the ferryl system of 3.7 kcal mol^{-1}, which suggests that *N*-dealkylation will largely be catalyzed by the low-spin pathway. The calculations did not favor an SET pathway, as it proceeded via a higher energy species. However, calculations always reflect assumptions built into the mechanisms that are analyzed—in this case, independent single electron transfer versus hydrogen abstraction from the carbon. Kinetic isotope effects were calculated for aniline bearing two CD_2H groups and the predicted isotope effects for oxidation by the compound I low-spin state matched reasonably well the experimentally observed low isotope effects. In a subsequent, but related study, ^{15}N-isotope effects were calculated for the oxidation of *N*-alkylamines by the compound I low- and high-spin states [143], which also favored a hydrogen abstraction mechanism. The low-spin state predicted normal secondary isotope effects, and the high-spin state inverse isotope effects, although these were not experimentally determined.

Contradictory evidence thus exists for SET and HAT pathways in the cytochrome P450-catalyzed *N*-dealkylation of amines, which suggests that both pathways may differentially contribute to *N*-dealkylation of specific substrates. It can be argued, however, that the two pathways are not actually independent of each other. As reported, the pK_a of cytochrome P450 compound II is ~12, whereas that of compound II of hemoproteins with an imidazole rather than thiolate iron ligand is in the range of 3–6 [85]. This very large difference in pK_a means that reduction of cytochrome P450 compound I to compound II is greatly facilitated in thermodynamic terms by protonation of the ferryl oxygen to give the Fe(IV)–OH species, whereas the corresponding intermediate in HRP, with its much lower pK_a value, is best written as Fe(IV)=O. The pK_a values of protons adjacent to the nitrogen radical cations of trimethylamine and dimethylaniline have been estimated to be ~15 and 9 [140, 144], well within the range of a ferryl oxygen with a pK_a of ~12 [85]. Green and colleagues have cogently argued that acceleration of hydrogen abstraction (the HAT reaction)

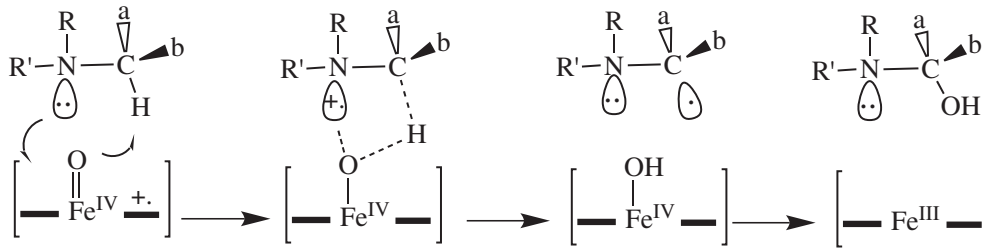

Fig. 4.20 A mechanism that exploits the high pK_a of cytochrome P450 compound II in the *N*-dealkylation of alkylamines. In this proposed mechanism, electron transfer from the nitrogen to compound I occurs concomitantly with transfer of a hydrogen from the adjacent carbon to the ferryl oxygen

in carbon hydroxylation by concurrent protonation of the compound II ferryl oxygen allows this reaction to compete with electron transfer from oxidizable residues in the protein, making possible normal P450 hydroxylation reactions [85]. Extrapolation of these arguments to *N*-dealkylation suggests either that a HAT pathway will be favored or, more likely, that electron transfer from the nitrogen to compound I is coordinated with abstraction of the hydrogen from the adjacent carbon. Such a proton-coupled electron transfer mechanism allows for different degrees of synchronicity of the two processes, giving rise to different isotope effects and electronic requirements. In contrast, an SET electron transfer mechanism is favored in the corresponding reaction catalyzed by HRP because (a) the thermodynamic gain due to protonation of the compound II ferryl oxygen is lost due to its much lower pK_a, and (b) sequestration of the ferryl oxygen in the active site hinders its direct interaction with substrate atoms [145, 146]. Finally, the oxidation of an *N*-alkylamide, in which electron transfer is much more difficult due to extensive delocalization of the nitrogen electron pair, approaches a limiting HAT mechanism. Schematically, the general mechanism can be envisioned to proceed via a transition state such as that in Fig. 4.20, with varying degrees of hydrogen abstraction accompanying the electron transfer step.

Stabilization of the carbon radical formed by removal of a hydrogen from an alkylamine is optimal when the C–H bond that is broken is aligned with the orbital that has the unpaired nitrogen electron pair or the positive charge in the nitrogen radical cation (Fig. 4.21). Structural or enzymatic constraints that influence this alignment will therefore play a role on the reaction specificity. Early studies demonstrated that chemically mediated *N*-dealkylation of alkylamines proceeding via the nitrogen radical cation favored the loss of an *N*-methyl over larger *N*-alkyl groups, such as an *N*-ethyl or *N*-isopropyl, because steric effects made it easier to properly align the C–H bond of the methyl with the nitrogen radical cation orbital [147]. Model studies of the oxidation of deuterated 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridines by *tert*-butoxyl radicals indicated that the reaction often resulted in removal of a hydrogen that had a considerably higher BDE despite the presence of a hydrogen with a weaker C–H bond strength. An analysis of this effect led to the conclusion that entropy factors associated with

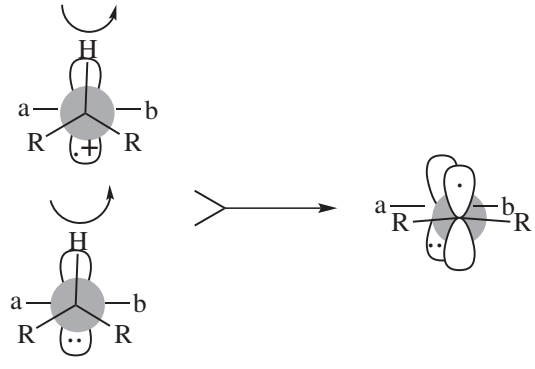

Fig. 4.21 Optimal alignment for hydrogen abstraction by the P450 ferryl species occurs when the hydrogen to be abstracted is aligned with the orbital holding the electron pair in the neutral nitrogen or the unpaired electron in the nitrogen radical cation

proper alignment of the C–H bond, the nitrogen electron pair, and the *tert*-butoxyl radical were of primary importance in determining the specificity and were responsible for the discrepancy between C–H bond strengths and reaction rates [148]. These effects may be responsible for the finding that the V_{max} for *N*-demethylation is often faster than that for *N*-deethylation. This applies particularly to amines that have both an *N*-methyl and *N*-ethyl substituent [149–151], or comparative V_{max} values in which K_m differences are suppressed [152], although at subsaturating substrate concentrations the preference for *N*-deethylation versus *N*-demethylation will be sensitive to both V_{max} and K_m differences.

4.4 Heteroatom Oxidation

As described in the preceding section, some *N*-dealkylation reactions may arise, at least in part, from an SET process in which an electron is initially removed from the nitrogen atom and therefore can be formally viewed as heteroatom oxidations. However, less cryptic examples of nitrogen oxidations in which the ferryl oxygen is added to the nitrogen instead of a vicinal carbon in the metabolic product are catalyzed by cytochrome P450 enzymes. These reactions are either hydroxylations in which, akin to hydrocarbon hydroxylations, an oxygen is inserted into an N–H bond, or heteroatom oxidations in which the immediate product is an *N*-oxide. The hydroxylation of *para*-substituted acetanilides is a good example of an *N*-hydroxylation that involves direct insertion of the ferryl oxygen into an N–H bond (Fig. 4.22a) [117, 153]. Nitrogen hydroxylations such as these are feasible because the nitrogen bears a hydrogen atom, its electron pair is highly delocalized into the amide carbonyl group and is therefore unavailable for oxidation, and there is no adjacent C–H bond.

On the basis of computational studies of the oxidation of aromatic amines that are carcinogenic, an alternative pathway for nitrogen hydroxylation has been postulated in which the ferric peroxy anion (Fig. 4.1f) deprotonates the nitrogen and the resulting nitrogen anion then

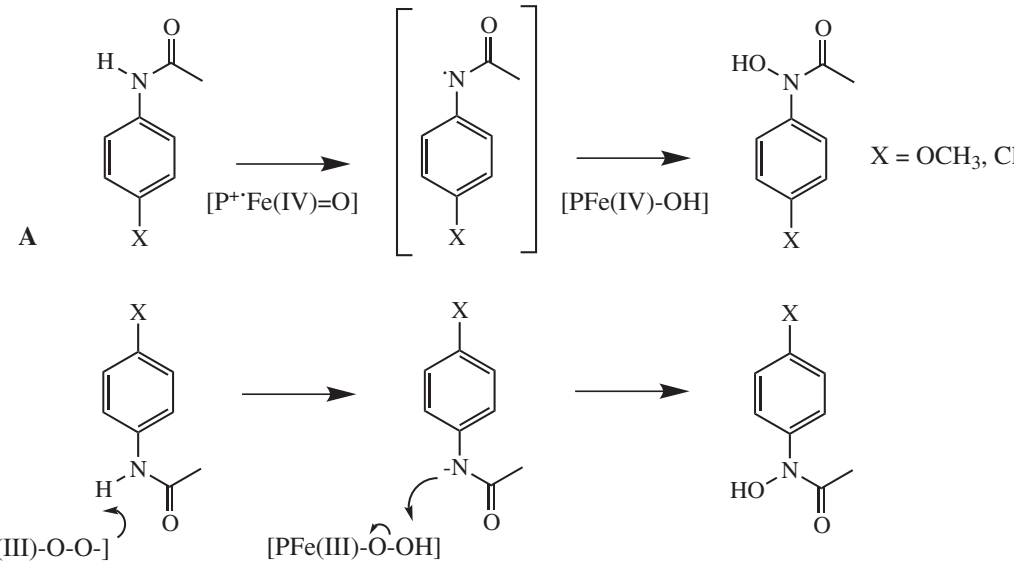

Fig. 4.22 Hydroxylation of the nitrogen of 4-substituted acetanilides, including phenacetin (X = OCH_3), is generally thought to occur by direct hydrogen abstraction from the nitrogen (**a**). However, computational results suggest an alternative in which a proton is removed from the nitrogen by the P450 ferric hydroperoxy anion intermediate, followed by nucleophilic attack on the ferric hydroperoxide by the nitrogen anion (**b**)

nucleophilically attacks the ferric hydroperoxide intermediate to generate the hydroxylamine. The primary support for this mechanism stems from the report that the peroxy anion mechanism (Fig. 4.22b) is computationally a better predictor of which aromatic amines will be carcinogenic [154, 155]. However, in contradiction, a related comparison of the mechanisms concluded that only the hydrogen radical abstraction mechanism (Fig. 4.22a) is viable [156].

Neutral nitrogen atoms in which the electron pair is not highly delocalized, or which do not have a hydrogen attached to them, can be oxidized by cytochrome P450 to the corresponding *N*-oxides. However, a comparison of the yields of *N*-dealkylation products versus *N*-oxides in the cytochrome P450-catalyzed oxidation of *p*-substituted *N,N*-dialkylanilines showed that, where both processes were unhindered, the primary reaction was *N*-dealkylation. Thus, the ratios of *N*-dealkylation to *N*-oxide formation in the CYP2B1-catalyzed oxidations of *N,N*-dimethylaniline and *N,N*-diethylaniline were 940 and 1020, respectively [149]. *N*-oxide formation can be viewed as involving electron abstraction from the nitrogen to give a radical cation, which then collapses with the iron-bound oxygen to give the *N*-oxide. In principle, this process should compete with *N*–dealkylation if it proceeds via an SET mechanism, as it involves the same intermediate. However, *N*-oxide formation does not appear to compete effectively with *N*-dealkylation in P450-catalyzed oxidations of nitrogen compounds unless (a) there are no hydrogen atoms on the carbons attached to the nitrogen, (b) a hydrogen atom is present on the carbon but is not properly oriented for abstraction, or is part of a strained ring system that limits conjugative interaction with the nitrogen atom, (c) the nitrogen bears at least one hydrogen and can be converted to a hydroxylamine, or (d) the nitrogen is in an environment that substantially lowers the energy for electron abstraction from the nitrogen. These limitations are consistent with the view that hydrogen abstraction occurs because the energy that is required for this process is lowered by some degree of concurrent proton transfer to the developing, highly basic compound II ferryl oxygen (Fig. 4.20). In agreement with this, *N*-oxides are significant metabolites in the oxidation of aromatic nitrogen heterocyles such as sorafenib [157], strained ring systems such as strychnine, which gives a stable α-hydroxylamine in addition to an *N*-oxide [158], and monoalkylamines such as mexiletine [159] (Fig. 4.23). However, it should be noted that the *N*-oxidation of simple alkylamines to *N*-oxides is readily catalyzed by flavin monooxygenases and is therefore not uncommon.

The oxidation of alkylthioethers by cytochrome P450 enzymes can produce both *S*-dealkylated and sulfoxide metabolites. As discussed for the oxidation of alkylamines, these transformations could result from a single two-electron reaction of the ferryl oxygen with the sulfur atom, a one-electron SET process that generates a sulfur radical cation as an intermediate, or, in the case of *S*-dealkylation, hydroxylation adjacent to the sulfur via a HAT mechanism. Analysis of substituent effects has shown that electron-donating groups increase the rate of oxidation of a thioether to a sulfoxide (Hammett $\sigma^+ = -0.16$) and of a sulfoxide to a sulfone (Hammett $\sigma^+ = -0.2$) [160,161]. Internal competition between the diarylthioether and symmetrically related diarylsulfone in thianthrene-5-oxide confirms the higher reactivity of the electron-rich thioether sulfur [162]. However, this result is expected and does not differentiate the possible mechanisms of sulfur oxidation. Efforts to determine if a sulfur radical cation is involved have generally been ambiguous. For example, the oxidation of phenyl cyclopropyl sulfide to its sulfoxide by a P450 enzyme from *Mortierella isabellina* occurs without detectable opening of the cyclopropyl ring (Fig. 4.24), but this may simply reflect a slower rate of ring opening than recombination to produce the sulfoxide [163]. Computational results indicate that sulfoxidation is mediated by the high-spin state of compound I, in contrast to *N*-dealkylation, for which oxidation by the low-spin state is preferred [142, 164]. Computational results also suggest that sulfoxidation is mediated by the compound I ferryl species rather than the ferric hydroperoxide that precedes it in the catalytic cycle [165]. However, comparison of the

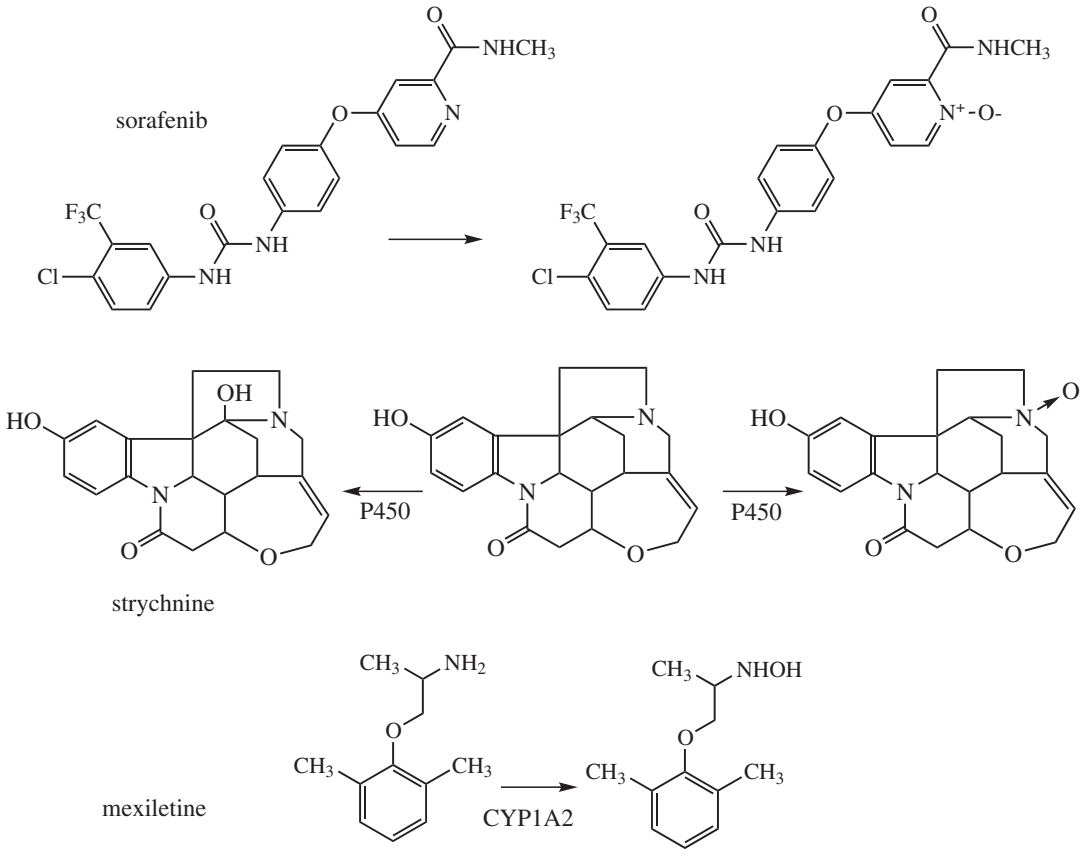

Fig. 4.23 Cytochrome P450 can oxidize ring-strained or aromatic nitrogens such as those of sorafenib and strychnine to *N*-oxides, but nitrogen hydroxylation is less common with alkylamines such as mexiletine

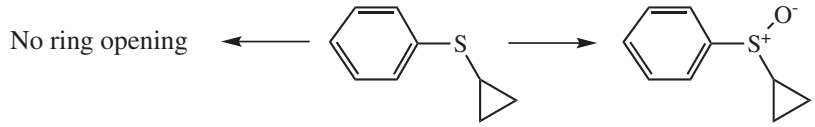

Fig. 4.24 Oxidation of a cyclopropyl-substituted thioether occurs without opening of the cyclopropyl ring, because either a sulfur radical cation is not formed or the cyclopropyl ring-opening reaction is too slow to compete with oxygen transfer to the sulfur

stereochemistry of CYP102-catalyzed fatty acid hydroxylation with that of sulfoxidation when a sulfur is substituted for the normally hydroxylated carbon in the fatty acid chain revealed that the absolute stereochemistry of sulfur oxidation was opposite to that of hydroxylation [166]. Furthermore, mutation of the conserved catalytic threonine (Thr238) to an alanine slowed hydroxylation, but had no effect on sulfoxidation. To explain this result, the authors postulated that the sulfur was oxidized by the ferric hydroperoxide intermediate, whereas carbon hydroxylation was mediated by the compound I ferryl species. If the interpretation is correct, these results suggest that the ferric hydroperoxide intermediate may occasionally contribute to sulfur oxidation.

Halide oxidation is generally not observed, as the halogen atoms are too electronegative to readily undergo P450-catalyzed oxidation. However, halogen oxidation has been implicated in

situations where alternative oxidation sites are sterically or chemically excluded. The clearest example of this is the oxidation of 12-chloro- and 12-bromododecanoic acids by both CYP4A1 and CYP52A21 (Fig. 4.25). These enzymes normally have a high preference for oxidation of fatty acids such as dodecanoic acid at the terminal carbon atom, a reaction specificity that requires protein structural constraints to suppress the energetically more favored oxidation of the secondary carbon at the adjacent (ω-1) position. When the terminal carbon is replaced by a chloride or bromide, there is some shift to oxidation of the ω-1 position to give the aldehyde. However, a large part of the reaction results in oxidation of the halogen (R–X) to the halonium ($R–X^+–O^-$) species that undergoes hydrolysis to replace the halogen by a hydroxyl group. As shown by studies with ^{18}O-labeled water, the hydroxyl group derives from the medium [167, 168]. In an earlier study, rat liver microsomes were shown to oxidize an iodoaryl compound to a product that is consistent with initial oxidation of the iodide to the iodoso ($RI^+–O^-$) state (Fig. 4.26) [169]. These examples indicate that halide oxidation is not beyond the oxidative capabilities of P450 enzymes, but is energetically difficult and exceedingly rare.

4.5 Olefin and Acetylene Oxidation

The cytochrome P450-catalyzed oxidation of nonaromatic carbon–carbon double bonds usually, but not always, results in formation of the corresponding epoxide. Epoxidation, as demonstrated by early experiments on the oxidation of olefins such as *cis*-stilbene [170], oleic acid [171], and *trans*-[1–^{2}H]-1-octene [172], invariably proceeds with retention of the olefin stereochemistry. To date, no example is known of a P450-catalyzed epoxidation that does not proceed with retention of stereochemistry. This retention of stereochemistry argues for a mechanism in which the transition state involves interactions of

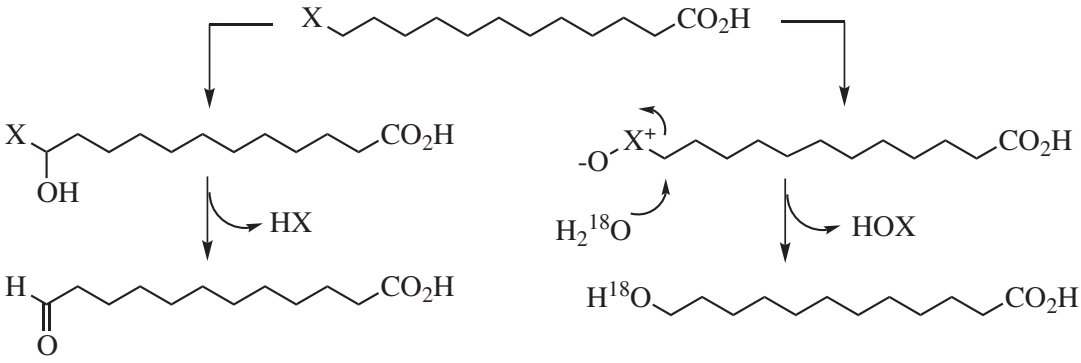

Fig. 4.25 The oxidation of 12-halododecanoic acids by CYP4A enzymes that normally oxidize the terminal methyl of fatty acids results in partial oxidation of the halogen atom to the halonium intermediate. This intermediate undergoes hydrolysis to produce the alcohol with incorporation of an oxygen from the medium

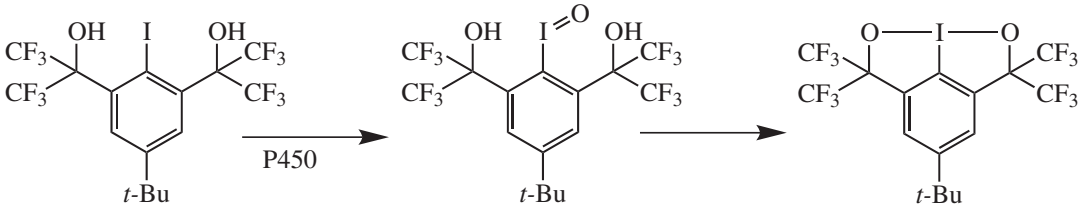

Fig. 4.26 Oxidation of the iodine in an aryl iodide by cytochrome P450

the ferryl oxygen with both carbons of the olefin, i.e., a "concerted" mechanism, although it does not require that both carbon–oxygen bonds be formed in a synchronous manner. Indeed, in early work, Hanzlik and Shearer reported differential effects of deuterium substitution on the two carbons of the olefinic double bond of *p*-methyl- and *p*-phenylstyrene, an inverse isotope effect ($k_H/k_D = 0.93$) being observed with deuterium on the internal carbon, but none when deuterium was on the terminal carbon [173]. If the two bonds had been formed simultaneously, one would have expected comparable inverse isotope effects with deuterium substitution on either carbon of the double bond.

Although most olefin oxidations appear to proceed via a synchronous mechanism to give the epoxides, strong experimental evidence for the oxidation of at least some olefinic bonds via a nonconcerted mechanism is provided by the occasional direct formation of carbonyl rather than epoxide products. Early work showed that trichloroethylene is oxidized to both trichloroethylene oxide and trichloroacetaldehyde [174, 175]. The demonstration that trichloroacetaldehyde did not derive from trichloroethylene oxide under the experimental conditions required that the two products be formed by distinct mechanisms (Fig. 4.27). Similarly, the oxidation of 1,1-dichloroethylene to monochloro- and dichloroacetic acids [176], of *trans*-1-phenylbutene to give 1-phenyl-1-butanone and 1-phenyl-2-butanone as minor products [176], and of styrene to 2-phenylacetaldehyde [177] does not appear to involve the epoxide as an intermediate. More recently, it has been shown that the 7,8-double bond of 7-dehydrocholesterol is oxidized by CYP7A1 directly to a 7-keto function without formation of the epoxide [178]. Deuterium substitution demonstrated that the C7 hydrogen migrates to the C8 position in this reaction (Fig. 4.28), in the same way that the oxidation of the chlorinated olefins involved shift of a hydrogen or a chloride to the adjacent carbon. These hydrogen and halide migrations implicate a cationic intermediate in the reaction, as shown in Fig. 4.27. This could result from a two-electron reaction with the oxygen that directly yields the cation, or could be envisioned as proceeding via an initial radical intermediate from which an electron is transferred to the ferryl species before the radical collapses with the ferryl oxygen to give the epoxide. There is no experimental evidence to differentiate these alternatives, although efforts to detect a radical intermediate, for example by searching for cyclopropyl ring-opened products in the oxidation

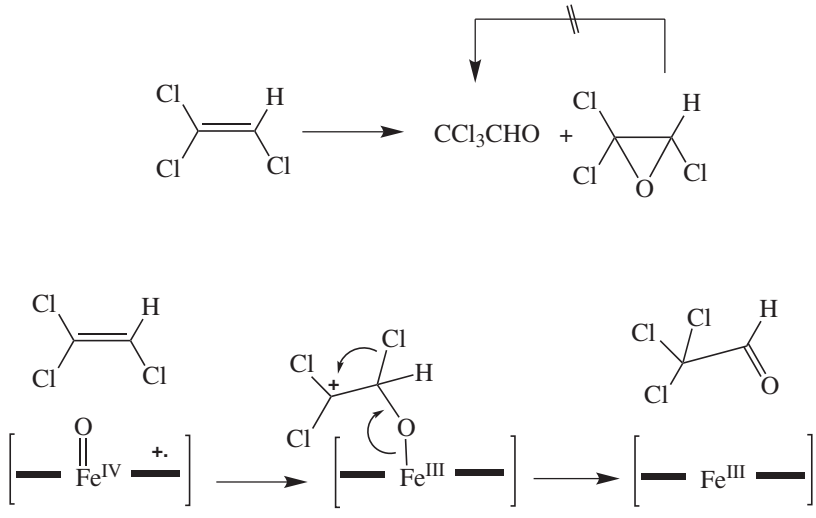

Fig. 4.27 The cytochrome P450-catalyzed oxidation of trichloroethylene yields not only the epoxide but also trichloroacetaldehyde, which is not formed under the same conditions from the epoxide and is therefore directly generated. A mechanism is proposed for this oxidative rearrangement

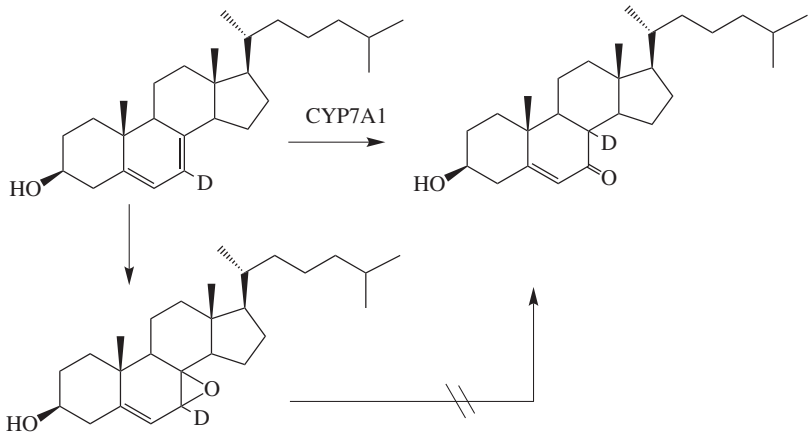

Fig. 4.28 Oxidation of the 7,8-double bond of 7-dehydrocholesterol directly produces the 7-ketone with migration of the 7-hydrogen to C8 by a mechanism that does not involve the 7,8-epoxide as an intermediate

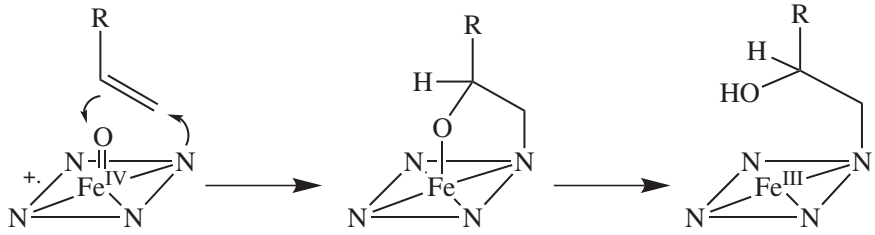

Fig. 4.29 Alkylation of a pyrrole nitrogen of the porphyrin heme framework occurs during the cytochrome P450-catalyzed oxidation of many terminal olefins. The heme porphyrin ring is represented by the square of pyrrole nitrogen atoms. The structure of the resulting adduct is shown, although which of the four porphyrin nitrogens is alkylated depends on the specific enzyme topology

of *trans*-1-phenyl-2-vinylcyclopropane, were not successful [179].

Independent evidence that olefin oxidation can proceed via a nonconcerted mechanism is provided by the fact that terminal olefins are not only oxidized to epoxides but, in many cases, simultaneously alkylate the P450 prosthetic heme group by covalently binding to one of its pyrrole nitrogen atoms (Fig. 4.29) [180]. It should be noted, however, that this heme alkylation process is relatively infrequent, with ratios of epoxidation to heme alkylation usually greater than 200. Despite the structures of the heme adducts, which nominally could arise by nucleophilic attack of the pyrrole nitrogen on the epoxide, epoxides are not involved in heme alkylation. This was definitely established by the fact that the synthetic epoxides do not react with the heme [181], and the observation that the stereochemistry of the heme adducts is not consistent with the backside attack of a nitrogen on the epoxide [182]. These results plus the fact that enzyme catalytic turnover is required and an atom from molecular oxygen is incorporated into the adduct indicate that heme alkylation is mediated by a transient intermediate formed during oxidation of the double bond by the enzyme [182–184].

DFT calculations suggest that the doublet- and quartet-spin states of the P450 compound I ferryl porphyrin radical cation are energetically close. According to these calculations, both the doublet and quartet species oxidize ethylene by addition of the ferryl oxygen to one carbon, leaving an unpaired electron on the second carbon of the double bond (Fig. 4.30) [185]. This intermediate radical also exists in doublet and quartet

states, although two equilibrating electromers exist for each of the two states, in one of which the electron from the original π-bond neutralizes the porphyrin radical cation and in the other in which it reduces the iron to the ferric state. In terms of product formation, the important difference between the doublet and quartet states is that closure of the doublet state to the epoxide is a barrierless process, resulting in an essentially concerted process even though computation does not predict a concerted mechanism with concurrent formation of both carbon–oxygen bonds [186]. This is not true for closure of the quartet state to the epoxide, for which a barrier from 2.3 to 7.2 kcal mol^{-1} is predicted [185]. Further DFT studies suggest that the activation energy, and thus the rate of substrate epoxidation, correlate with the ionization potential of the olefin, electronic properties of the oxidizing agent such as its polarizability volume, the electron affinity of the

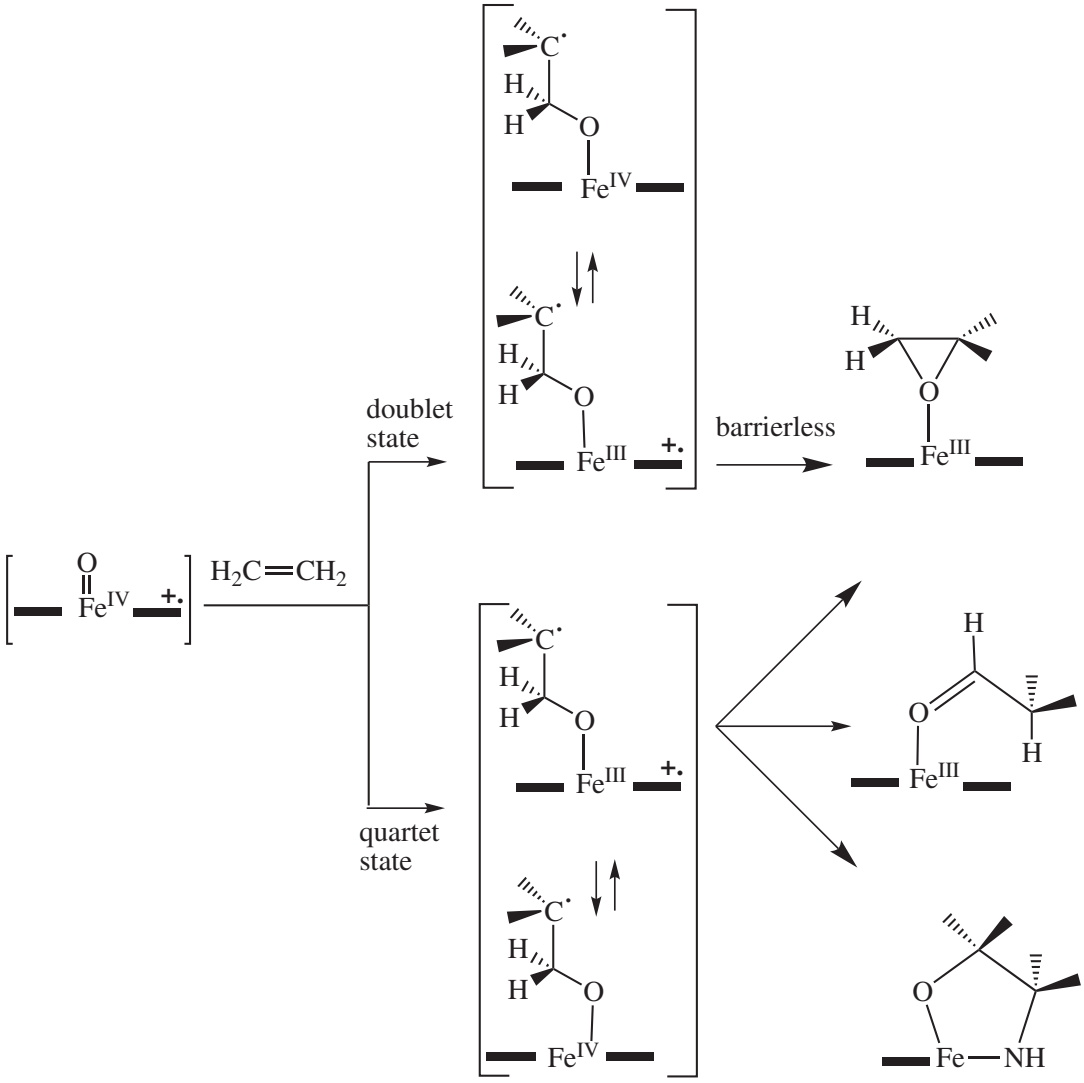

Fig. 4.30 Schematic outline of the oxidation of a double bond by the two different electromeric spin states of cytochrome P450 compound I. The compound I doublet state produces the epoxide via an essentially barrierless transition state. On the other hand, the quartet state proceeds via a discrete radical intermediate that allows competing reactions leading to substrate rearrangement or heme alkylation to occur

oxidizing species, and the strength of the newly formed C–O bond [187, 188]. The energy barrier in the epoxidation pathway for the quartet state makes alternative reactions, such as direct carbonyl formation or heme alkylation, competitive processes [189, 190]. According to this scenario, the ratio of epoxidation to alternative reactions is largely governed by the ratio of the doublet and quartet transition states. It also implies the existence of a radical state in the catalytic trajectory for the oxidation of olefins.

Acetylenes, which have shorter and stronger π-bonds than olefins, can also be oxidized by cytochrome P450 enzymes. The oxidation of terminal acetylenes gives ketenes in which the terminal hydrogen has quantitatively migrated to the internal carbon of the triple bond (Fig. 4.31) [191, 192]. The ketene is then hydrolyzed to yield the carboxylic acid as the observed metabolite. By analogy to the oxidation of olefins, the immediate product should be the unsaturated epoxide (oxirene), but oxirenes are extremely unstable structures that are difficult to detect even at cryogenic temperatures in chemical experiments. It is therefore almost certain that oxirenes are not actual intermediates in the oxidation, and thus that migration of the terminal hydrogen occurs during the oxidation step to directly yield the ketenes. Support for this inference is provided by the fact that major kinetic isotope effects have been observed in the oxidation of deuterium-substituted aryl acetylenes to arylacetic acids [192, 193]. The oxidation of disubstituted triple bonds is much less common than that of terminal acetylenic groups, but is not unknown. Thus, the CYP1A1- and CYP1A2-catalyzed formation of 1-biphenylpropionic acid as a minor product from 4-(1-propynyl)biphenyl involves oxidation of the triple bond with concurrent migration of an alkyl group rather than a hydrogen [194]. Migration of a chloride atom in the in vivo oxidation of dichloroacetylene to give dichloroacetic acid as a minor product has also been reported [195].

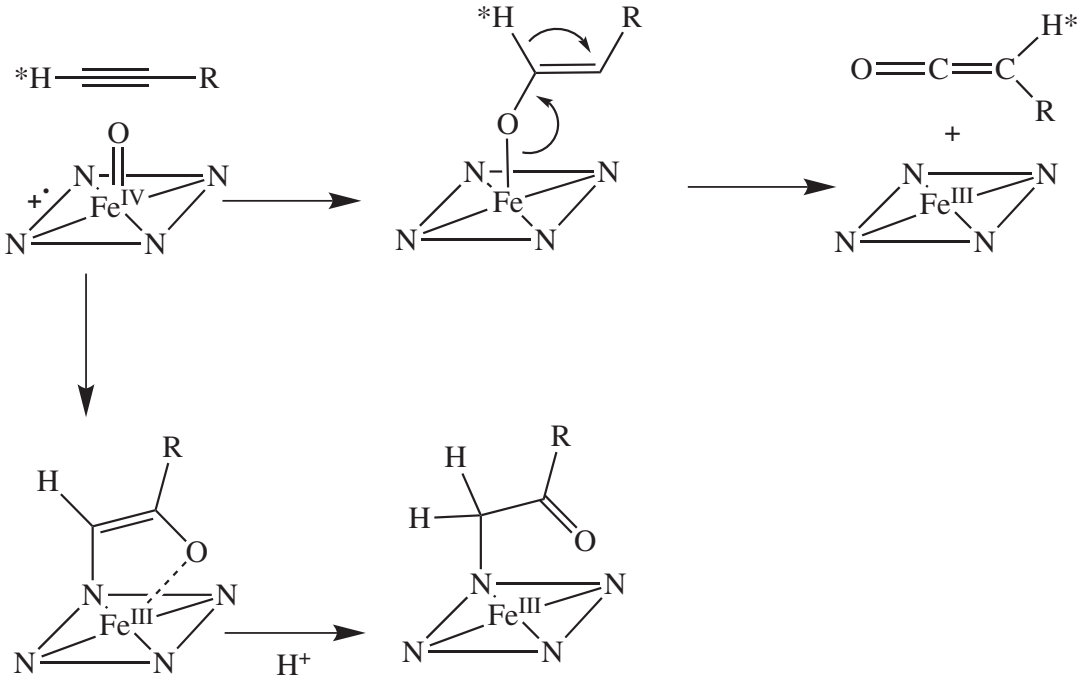

Fig. 4.31 The oxidation of terminal acetylenes in which the oxygen is added to the terminal carbon produces ketene metabolites in which the acetylenic hydrogen (*H**) has migrated to the adjacent carbon. If the oxidation involves addition of the ferryl oxygen to the internal carbon of the triple bond, heme alkylation occurs

The oxidation of terminal acetylenes, like that of monosubstituted olefins, often results in inactivation of the P450 enzyme involved in the oxidation. In some instances, this inactivation involves reaction of the ketene metabolite with nucleophilic residues on the protein [196, 197], but in other instances it involves alkylation of the prosthetic heme group (Fig. 4.31). Again, as found for heme alkylation in the oxidation of olefins, the terminal carbon of the acetylene binds to a pyrrole nitrogen of the heme and a hydroxyl is attached to the internal carbon of the triple bond. Of course, as one of the two π-bonds of the acetylene remains in the adduct, keto–enol equilibration yields a final adduct structure with a carbonyl on the original internal carbon of the triple bond [182, 198]. It is to be noted that the oxidation of terminal triple bonds that produces ketene metabolites requires addition of the ferryl oxygen to the unsubstituted, terminal carbon, whereas the oxidation that results in heme alkylation requires its addition to the internal carbon. As a rule, the ratios of metabolite formation to heme alkylation are much smaller for terminal acetylenes than for olefins.

4.6 Aromatic Ring Oxidation

The cytochrome P450-catalyzed introduction of a hydroxyl group into an aromatic ring is generally known as an aromatic hydroxylation, but mechanistically involves reaction of the ferryl species with the aromatic π-system rather than with the C–H bond. The C–H BDE of benzene, is 112 kcal mol^{-1} [199], much higher than the BDE of ~89–100 kcal mol^{-1} of alkyl C–H bonds (Table 4.2). This high-energy barrier makes direct oxygen insertion into an aromatic C–H bond energetically difficult. Aromatic hydroxylation is therefore mechanistically related to P450 olefin oxidation rather than carbon hydroxylation.

In its original formulation, aromatic ring oxidation yields an unstable epoxide that readily rearranges by heterolytic cleavage of one of the epoxide carbon–oxygen bonds, presumably assisted by hydrogen-bonding interactions, to give a resonance-stabilized cation (Fig. 4.32). The cation is then neutralized by migration of a hydrogen anion from the carbon that bears the newly introduced oxygen. Enolization of the resulting

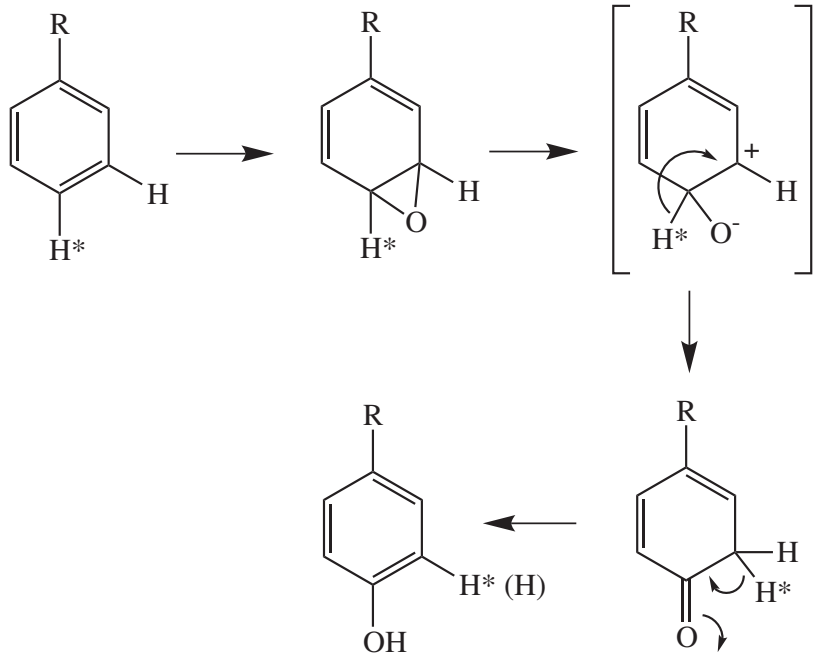

Fig. 4.32 The classic NIH shift involves epoxidation of an aromatic ring, heterolytic opening of the epoxide with migration of a hydrogen atom (*H**) to the resulting cationic carbon, and enolization of the resulting ketone to regenerate the aromatic ring

ketone, which is thermodynamically favored because it regenerates the aromatic ring, produces the final phenolic structure. In the enolization step, either the original hydrogen or the one that migrated to the carbon (labeled H*) is lost, which explains why the migrating hydrogen is only partially retained in the product. This sequence was formulated by investigators at the National Institutes of Health and is therefore termed the "NIH-shift" [200]. In most cases, epoxidation of aromatic rings occurs at an unsubstituted π-bond, so the migrating atom is a hydrogen, but the oxidation of sites in which one of the two carbons bears a halide or alkyl group, resulting in migration of the halide or alkyl moiety, is known [200, 201].

The deuterium-sensitive keto–enol tautomerization occurs after the rate-limiting step in which the ferryl oxygen adds to the aromatic ring, so the rate of aromatic hydroxylation is not subject to primary isotope effects on deuterium substitution. However, small inverse secondary deuterium isotope effects (0.83–0.94) have been observed in the aromatic hydroxylation of *ortho*- and *para*-xylene, a finding in agreement with rate-limiting addition of the ferryl oxygen to a π-bond, as this requires partial rehybridization from the sp^2 to the sp^3 state of at least one of the two carbons of the π-bond [202]. These inverse isotope effects are inconsistent with an alternative mechanism in which the aromatic ring transfers an electron to the ferryl species in the rate-determining step to produce a π-radical cation intermediate, as this would entail minimal rehybridization of the carbons.

The formation of epoxides in the P450-catalyzed oxidation of aromatic rings has been directly demonstrated, for example in the oxidation of benzene [203], or can be inferred from isolation of subsequently formed *trans*-dihydrodiol or glutathione conjugates, of which there are many examples, e.g., phenanthrene [204]. However, epoxide metabolites are not mandatory intermediates in the oxidation of aromatic rings. One example is provided by hydroxylations, often *meta* to a halide substituent, in which the hydrogen on the hydroxylated carbon is quantitatively lost, showing no NIH shift has occurred, and for which a small primary deuterium isotope effect is observed [205, 206]. Further studies with deuterated substituted benzenes revealed a small, normal isotope effect ($k_H/k_D = 1.1–1.3$) for *meta*-hydroxylation of chlorobenzene with a deuterium at the hydroxylated *meta*-position, in contrast to a small, inverse isotope effect of $k_H/k_D = \sim 0.95$ for *ortho*- and *para*-hydroxylation when the deuterium was at those positions [207, 208]. These results suggest that *meta*-hydroxylation occurs by a different mechanism than *ortho*- or *para*-hydroxylation, for which the isotope effects are consistent with epoxide formation in the rate-limiting step. The mechanism that is proposed for these hydroxylations, *ipso*-substitution, postulates the formation of a carbon–oxygen bond with the ferryl oxygen, but one that, instead of closing to the epoxide, undergoes proton loss to directly give the hydroxylated aromatic ring (Fig. 4.33). However, it is likely that *ipso*-substitution and epoxide formation are simply two outcomes of a common reaction manifold in which the intermediate formed by addition of the ferryl oxygen to one of the carbons of the aromatic system can either close to an epoxide or undergo some form of *ipso*-substitution without epoxide formation (Fig. 4.33). Aromatic oxidation thus parallels the scenario for olefin epoxidation in which a radical intermediate is formed that can either close to the epoxide or undergo alternative reactions, such as a hydrogen shift or addition to a heme nitrogen atom. Indeed, density functional calculations suggest that aromatic oxidation proceeds via addition to the ring to give a tetrahedral intermediate with radical and cation character, although cationic character may predominate in the enzymatic reaction. Subsequent rearrangement to give epoxide, ketone, and phenol products occurs by reactions with relatively low-energy barriers [209].

Direct oxidation of polyhalogenated aromatic compounds to phenols or quinones, or *para*-substituted phenols to quinones, is thought to occur by a variant of *ipso*-substitution. Pentafluorochlorobenzene is thus oxidized to tetraflu-

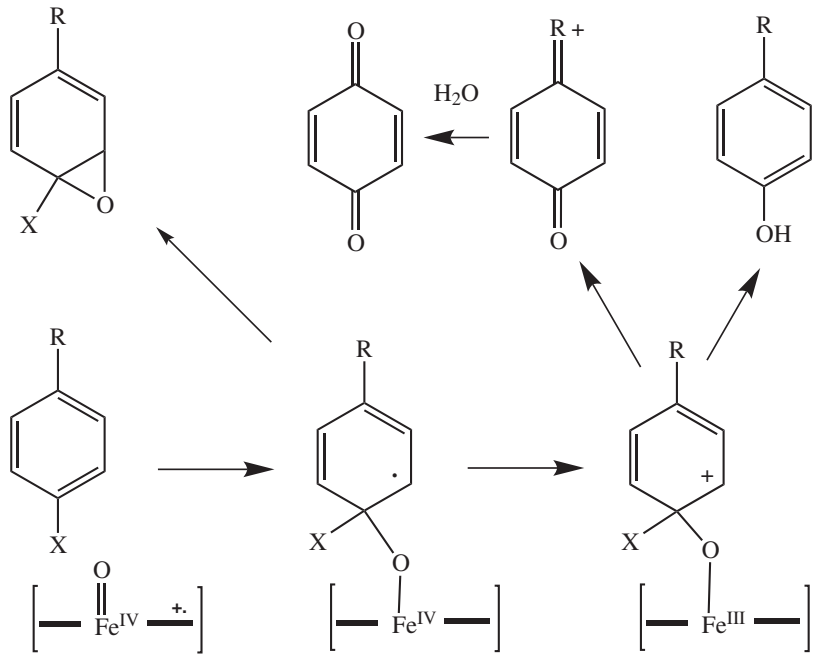

Fig. 4.33 A more generalized view of aromatic oxidation parallels that formulated for olefin epoxidation. Reaction of the compound I ferryl with the aromatic ring yields a radical intermediate that can close to the epoxide (giving rise to the classical NIH shift), but can also undergo alternative reactions that depend on the other substituents. In this figure, *X* is a hydrogen or a leaving group such as a halide or ether oxygen

orochlorophenol by addition of the P450 ferryl oxygen to the fluoro-substituted carbon *para*- to the chloride atom, with electron donation from the chloride leading to elimination of the fluoride (Fig. 4.34). The resulting chloronium cation then undergoes hydrolysis to generate tetrafluoroquinone, or reduction to produce tetrafluorophenol [210]. The regiochemistry of the oxidation of 1-fluorobenzene, 1,2-difluorobenzene, 1,3-difluorobenzene, 1,2,3-trifluorobenzene, and 1,2,4-trifluorobenzene, supported by molecular orbital calculations, indicates that the reaction proceeds by ferryl oxygen addition to the aromatic π-system rather than initial electron abstraction from the aromatic ring to generate a radical cation [211]. Furthermore, local density approximation calculations argue that in the oxidation of fluorobenzene to 4-fluorophenol, the NIH shift occurs from the initial tetrahedral intermediate without actual formation of the epoxide [212]. In a variant of these mechanisms, one computational study of the oxidation of rings such as hexachlorobenzene suggests that the tetrahedral intermediate collapses with migration of a chloride to the adjacent carbon to give an α,α-dichloroketone intermediate [213].

The P450-catalyzed oxidation of 4-substituted phenols to the hydroquinone occurs with loss of the *para*-substituent in a reaction that incorporates one atom of labeled molecular oxygen into the product (Fig. 4.35) [214, 215]. Based on the finding that converting the phenol to a methyl ether suppressed the reaction, it was proposed that one-electron oxidation of the phenol to the phenoxy radical was followed by combination with the compound II ferryl oxygen to give a tetrahedral intermediate that directly eliminates the substituent to form a second carbonyl group. Computational analysis of the aromatic ring hydroxylation of dopamine by CYP2D6 supports a

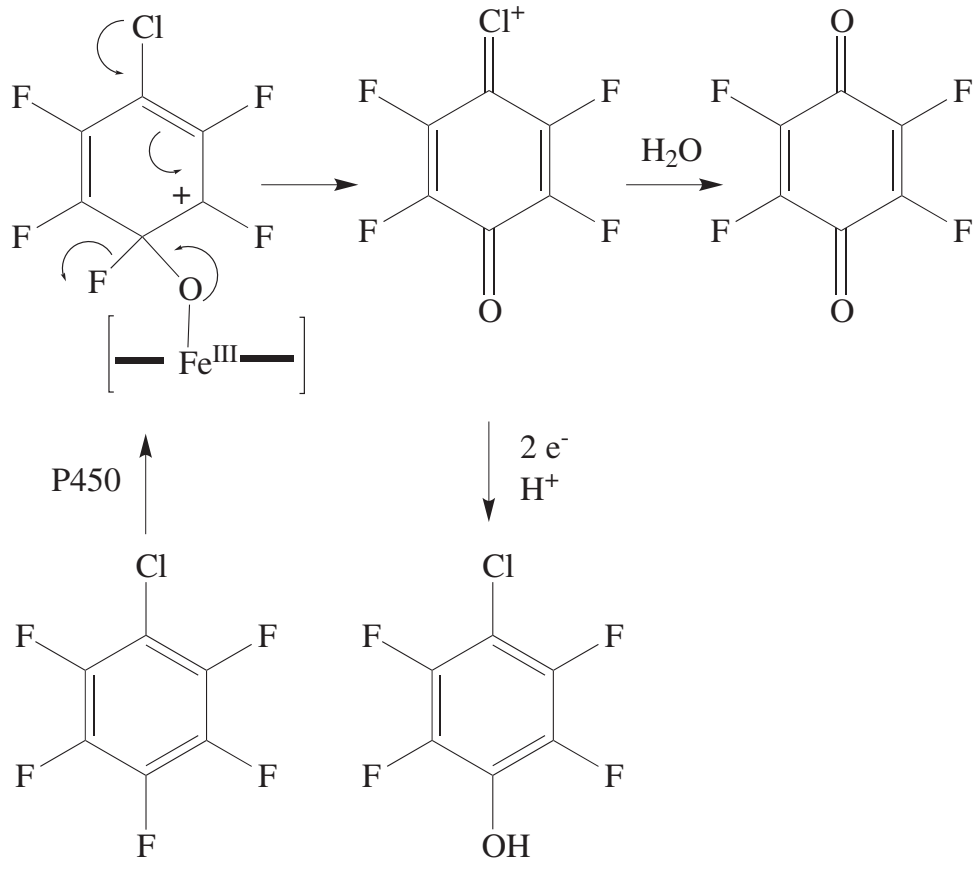

Fig. 4.34 *Ipso*-oxidation of chloropentafluorobenzene to phenol and quinone products by cytochrome P450

mechanism in which the formation of a phenoxy radical is followed by collapse of the radical with the ferryl oxygen [216]. 4-Iodoanisole, with a methoxy rather than phenol group, is reportedly oxidized to several metabolites, including 4-methoxyphenol, without the incorporation of an oxygen from water [217]. Aromatic oxidation by the *ipso*-mechanism can therefore also occur in the absence of a phenol group. Interestingly, the oxidation of 4-methylphenol, in which the methyl is not a leaving group, yielded 4-hydroxy-4-methyl-2,5-cyclohexadiene-1-one (Fig. 4.35) [214]. This result, however, does not distinguish between *ipso* addition and epoxidation, as both could produce the dienone product.

Independent evidence for the cytochrome P450-catalyzed oxidation of phenols to phenoxy radicals is provided by the growing number of plant and fungal P450 enzymes shown to catalyze the dimerization of phenols in the biosynthesis of natural products. Examples are the conversion of (*R*)-reticuline to salutaridine by CYP719B1 in morphine biosynthesis [218, 219], (*S*)-salutaridine to (*S*)-corytuberine by CYP80G2 in magnoflorine biosynthesis [220] and autumnaline to isoandrocymbine in colchicine biosyn-

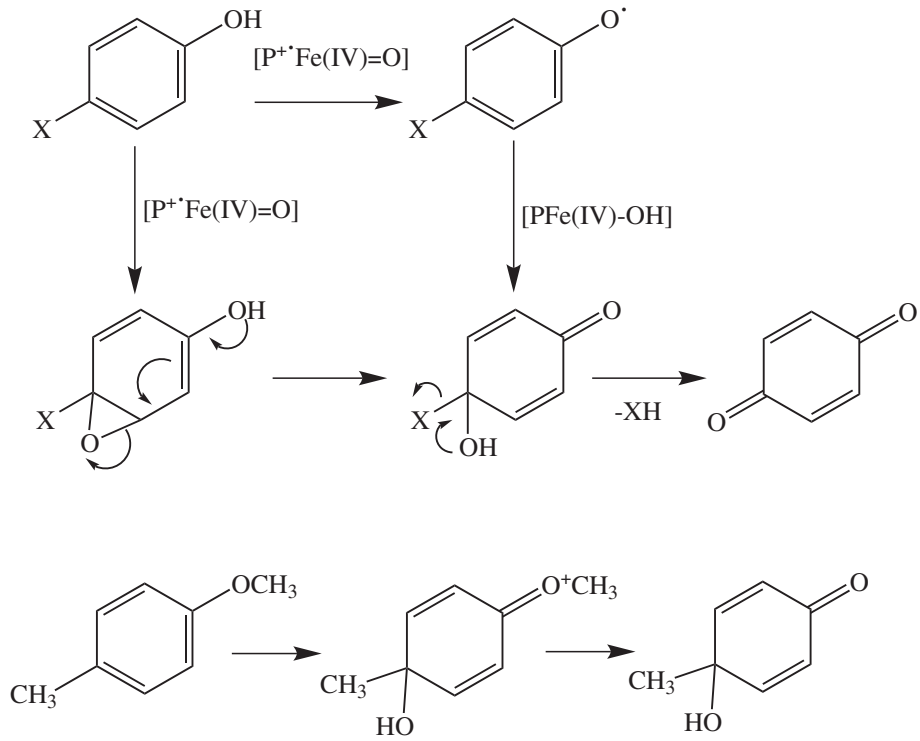

Fig. 4.35 Oxidation of 4-substituted phenols to quinones by cytochrome P450. If the 4-substituent is not a leaving group, as in 4-methylanisole, the product isolated is the keto-alcohol retaining the 4-substituent

thesis (Fig. 4.36) [221]. A beautiful and complex example is provided by the sequence of three phenol–phenol coupling reactions, catalyzed by different P450 enzymes, that produce vancomycin from an acyclic precursor (Fig. 4.37) [222, 223]. A cytochrome P450 enzyme, GstF, catalyzes a cyclization reaction in the biosynthesis of griseofulvin that produces a spiro-fused ring system (Fig. 4.38) [224]. An alternative mechanism was proposed involving initial epoxidation of the aromatic ring, but this mechanism is less attractive as it entails questionable reaction steps. A cytochrome P450 enzyme, JulI, from *Streptomyces* catalyzes the dimerization of nonaketide monomeric phenol units to produce the dimeric julichrome, setomimycin, and spectinomycin products [225]. It has also been reported that CYP3A4 and other mammalian enzymes can catalyze phenol-coupling reactions, including the oxidation of (*R*)-reticuline to salutaridine [226, 227], dimerization of the phenolic drug raloxifene [228], and dimerization of 17β-estradiol and estrone (Fig. 4.39) [229]. It is important in evaluating these dimerization reactions to rule out incidental peroxidative phenol coupling supported by H_2O_2 generated by the cytochrome P450–cytochrome P450 reductase system. This was done, for example, in the case of raloxifene dimerization, but not in that of estradiol dimerization.

In a related but different vein, the cytochrome P450 enzyme StaP (CYP245A1) catalyzes a putative diradical coupling reaction involving one-electron oxidation of each of two indole rings to form the indolocarbazole alkaloid skeleton of staurosporine and rebeccamycin (Fig. 4.40) [230, 231]. The crystal structure of the protein–sub-

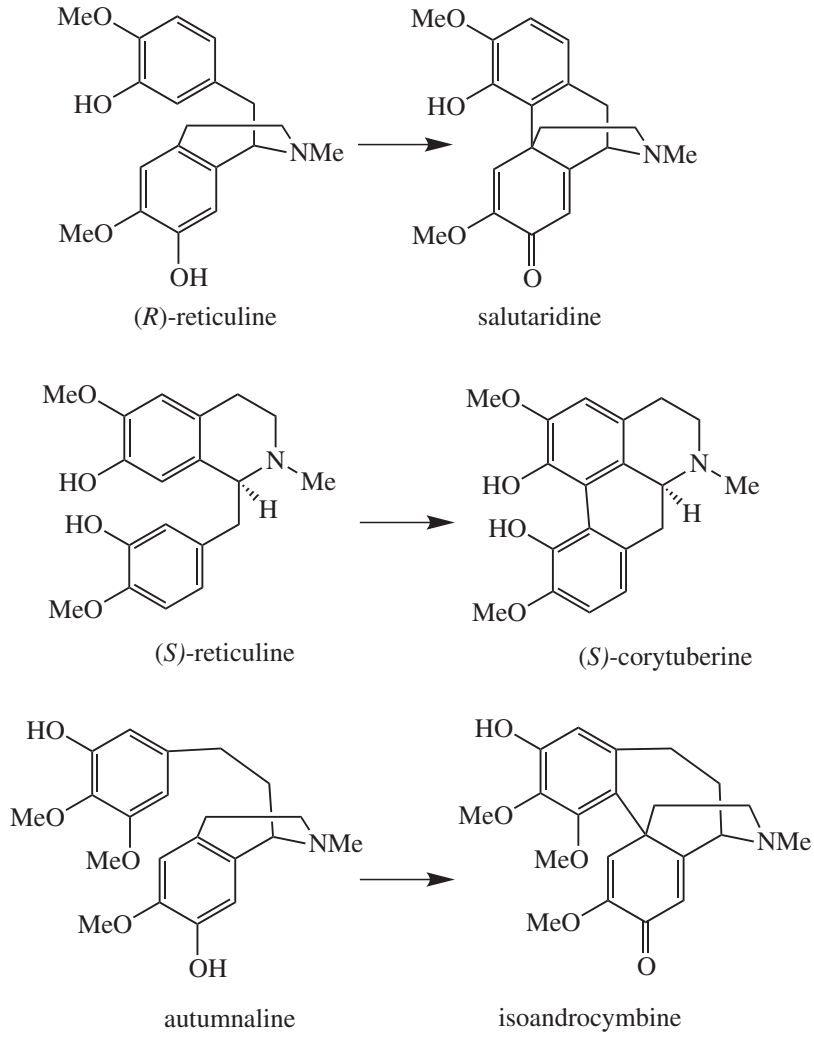

Fig. 4.36 Cytochrome P450-catalyzed phenol–phenol cross-linking reactions in the biosynthesis of the alkaloids salutaridine, (*S*)-corytuberine, and isoandrocymbine. These reactions presumably proceed via one-electron oxidation of each of the phenol groups followed by diradical coupling

strate complex placed the substrate at too great a distance for direct interaction with the ferryl oxygen, but computational analysis suggested that water molecules in the active site could facilitate the electron and proton transfers required for the coupling reaction [232]. The biosynthesis of the diketopiperazine alkaloid tryptophenaline by the P450 enzyme DtpC from *Aspergillus flavus* has been postulated to involve hydrogen abstraction from an amide nitrogen to give the nitrogen radical, cyclization of the radical with the attached indole ring, and finally carbon–carbon bond formation from two of the resulting radicals to give the dimeric product (Fig. 4.41) [233]. This figure requires that two molecules of the substrate be bound in the active site, both of which undergo oxidation to the initial nitrogen radical.

In summary, aromatic hydroxylation occurs via reaction of the aromatic π-electrons with the compound I ferryl oxygen to give transient tetra-

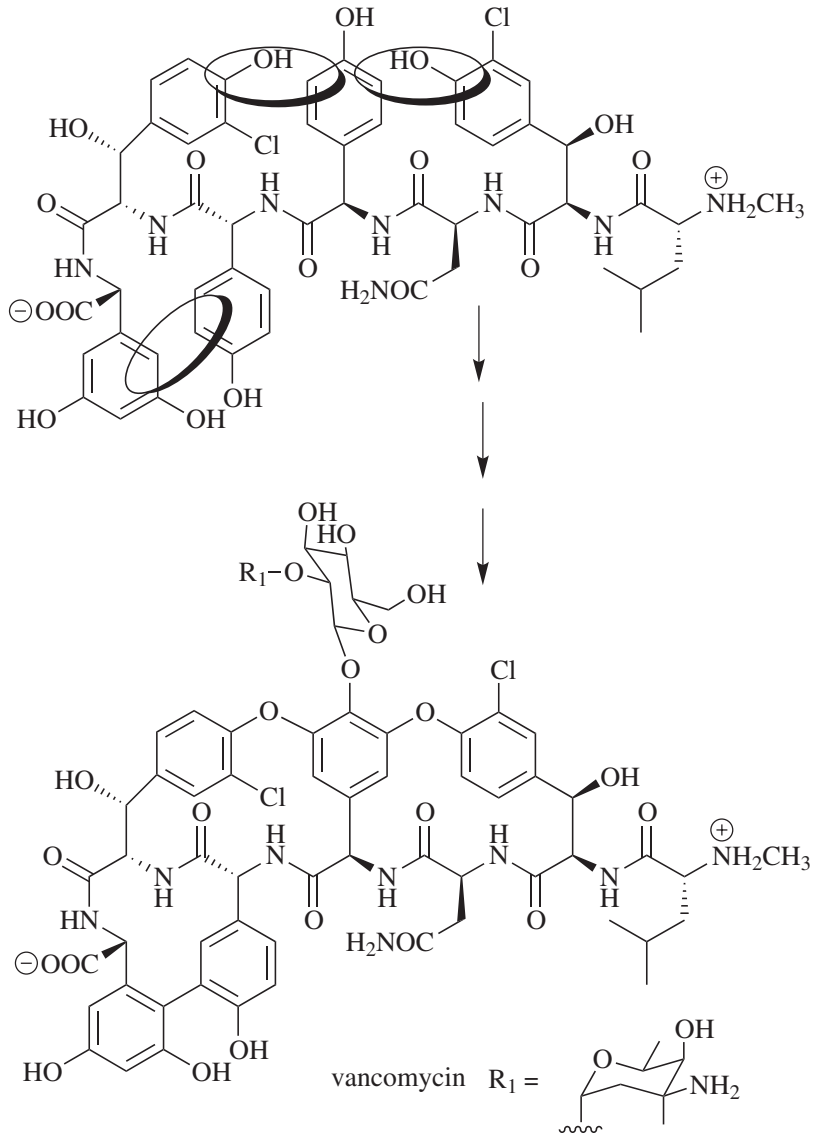

Fig. 4.37 Formation of three cytochrome P450-catalyzed phenol–phenol cross-links in the biosynthesis of vancomycin. The cross-linking sites are *circled*

hedral radical or cationic intermediates, which in turn collapse to the epoxide or undergo an *ipso*-substitution mechanism to give products that do not derive from the epoxide. In the presence of electron-donating groups on the aromatic ring, such as hydroxyl or amino functions, participation of these groups in determining the outcome of the aromatic oxidation is observed.

4.7 Carbon–Carbon Bond Cleavage

The cytochrome P450-catalyzed cleavage of a carbon–carbon (C–C) bond has long been of interest because of the key role this transformation plays in the biosynthesis of cholesterol and all the sterol hormones derived from it. These reactions include the 14α-demethylation of lanosterol by CYP51, truncation of the cholesterol side chain

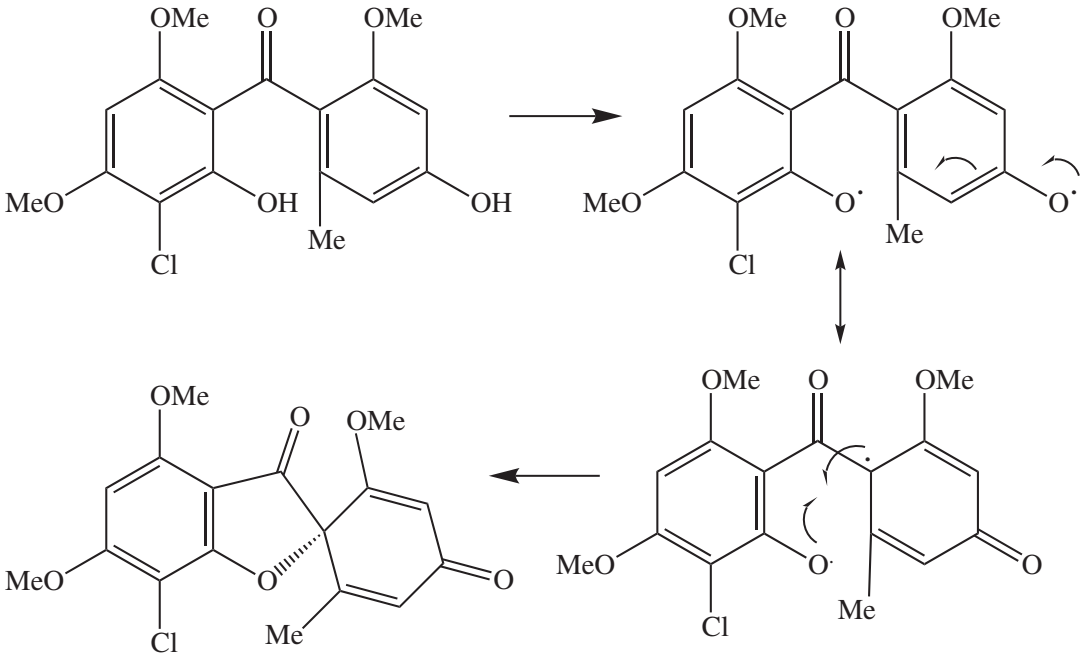

Fig. 4.38 Spirocyclization catalyzed by cytochrome P450 GstF in the biosynthesis of a precursor of the antibiotic griseofulvin showing the probable diradical intermediate involved in the coupling reactions

at C21–C22 to give pregnenolone by CYP11, replacement by CYP17 of the remaining side-chain fragment by an oxygen, and aromatization of androstenedione to estrogen by CYP19. All these cytochrome P450 enzymes undergo multiple sequential hydroxylations that first generate the required functionality and then promote the C–C bond cleavage. The sterol biosynthetic C–C bond cleavage reactions fall into three groups: (a) cleavage of a C–C bond between a carbonyl group and an adjacent carbon, (b) cleavage of a C–C bond between a hydroxyl and a ketone, and (c) cleavage of a C–C bond between two hydroxyl groups. However, the range of enzymes, substrates, and reactions that result in C–C bond cleavage continues to expand and diversify.

4.7.1 Cleavage Alpha to a Carbonyl Group

4.7.1.1 CYP51 (Sterol 14α-Demethylase)

CYP51, the first cytochrome P450 enzyme in the sterol biosynthetic pathway, removes the 14α-methyl group from sterols such as lanosterol and ergosterol by a process that introduces a C14–C15 double bond. This transformation involves three sequential catalytic events: The first is hydroxylation of the methyl group, the second oxidation of the resulting alcohol to an aldehyde, and finally, in the climatic third event, elimination of the oxidized methyl group as formic acid (Fig. 4.42) [234, 235]. The first two hydroxylations are conventional hydroxylations and require no discussion [236–238]. The more unusual step in which the carbon–carbon bond is cleaved is thought to occur by nucleophilic addition of the ferric hydroperoxy anion (Fig. 4.1f) to the aldehyde group, followed by a Baeyer–Villiger-like fragmentation of the resulting peroxyhemiacetal. Finally, the formyl intermediate thus obtained is eliminated as formic acid with introduction of the double bond (Fig. 4.43, path a). This mechanism is supported by labeling studies showing that the formic acid incorporates an oxygen atom from molecular oxygen in addition to the oxygen of the original aldehyde [239]. Furthermore, the proposed 14α-formyl intermediate has been iso-

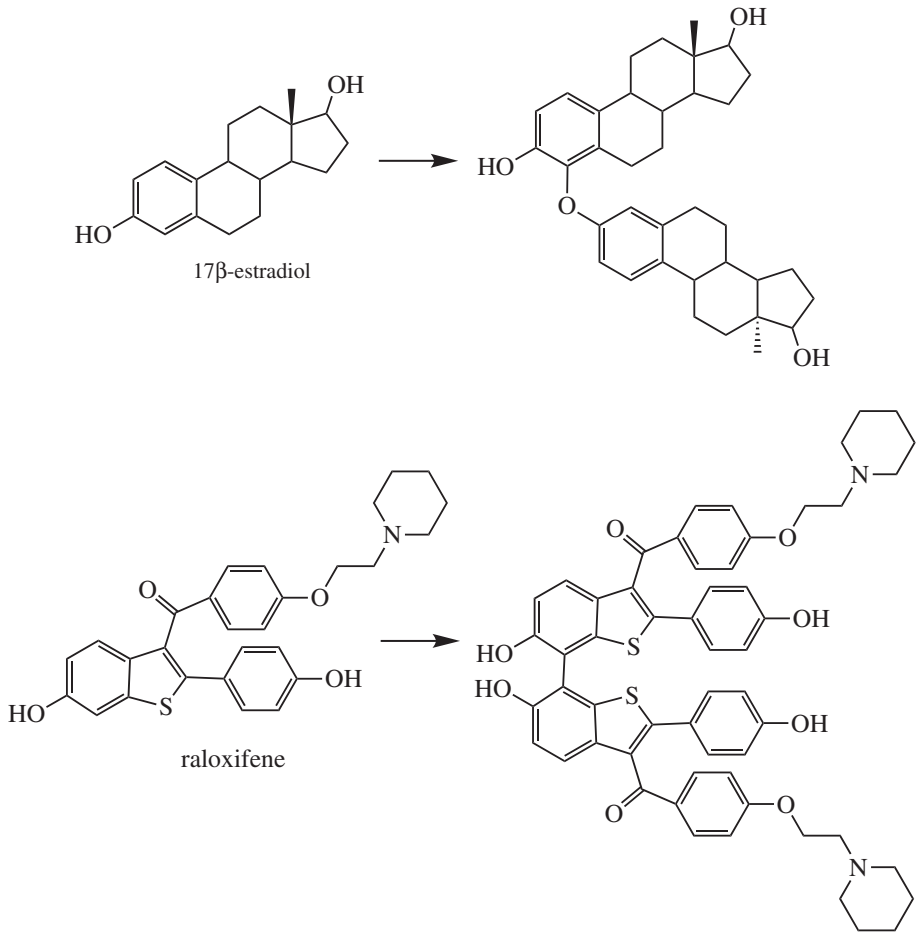

Fig. 4.39 The reported cytochrome P450-catalyzed dimerizations of estradiol and raloxifene

lated and spectroscopically characterized [240]. The formyl elimination step, which proceeds with stereospecific loss of the 15α-hydrogen that is located on the same face of the sterol framework, introduces the C14–C15 double bond [241, 242]. Several crystal structures of CYP51, including one of the full-length *Saccharomyces cerevisiae* enzyme with lanosterol bound to the protein [243], have been determined, but they do not shed much additional light on the catalytic mechanism.

It is not possible with the available evidence to exclude an alternative mechanism for the deformylation reaction that involves one-electron fragmentation of the peroxyhemiacetal intermediate, producing a free radical at C14. Transfer of one electron to the compound II ferryl species would then generate a cation that either undergoes immediate proton loss to give the C14–C15 double bond or, to a small extent, is trapped by the formate molecule (Fig. 4.43, path b). This mechanism offers a simple route to proton loss and double bond formation and is, perhaps, more consistent with the homolytic mechanisms that have been proposed for other sterol C–C bond cleavage reactions (see below).

4.7.1.2 CYP19 (Aromatase)

A demethylation concomitant with aromatization of the sterol A-ring occurs in the CYP19-catalyzed conversions of androstenedione and testosterone to estrone and estradiol, respectively

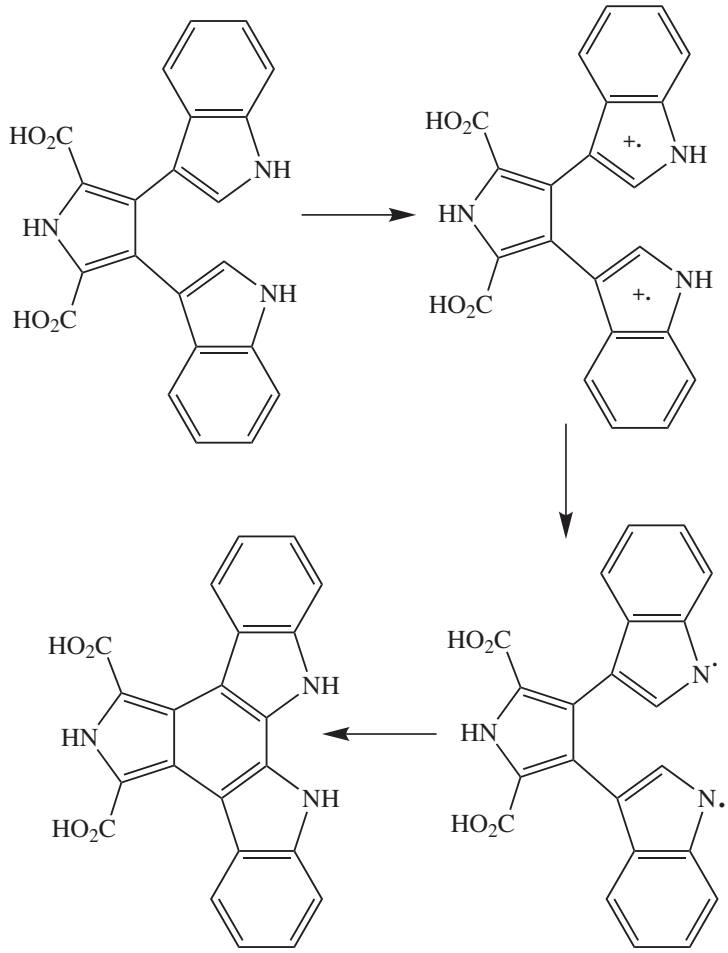

Fig. 4.40 Intramolecular cross-linking of two indole units in the biosynthesis of indolocarbazole alkaloids. Dimerization is most reasonably explained by the cytochrome P450-catalyzed generation of a radical on each of the two indole rings, as shown

[244, 245]. As with CYP51, the first two steps of the catalytic sequence are conventional hydroxylations that produce the 19-hydroxymethyl derivative and then, via a second stereospecific hydroxylation [246, 247], a 19-*gem*-diol that decays to the aldehyde (Fig. 4.44). In the final C–C bond-cleaving step of the catalytic sequence, the 1β and 2β hydrogens of the A-ring are lost and the C19 carbon is extruded as formic acid in which both oxygens derive from molecular oxygen [248, 249]. Kinetic analysis of the reaction and its intermediates is consistent with this sequence of events [250]. Over the years, a variety of mechanisms have been advanced to rationalize this C–C bond cleavage reaction, including the intervention of a 4,5-epoxide [251], 1β-hydroxyl [252], 2β-hydroxyl [253, 254], or C19 peroxide [248, 255]. In the currently most cited mechanism [256, 257], the ferric hydroperoxy catalytic intermediate (Fig. 4.1f) adds as a nucleophile to

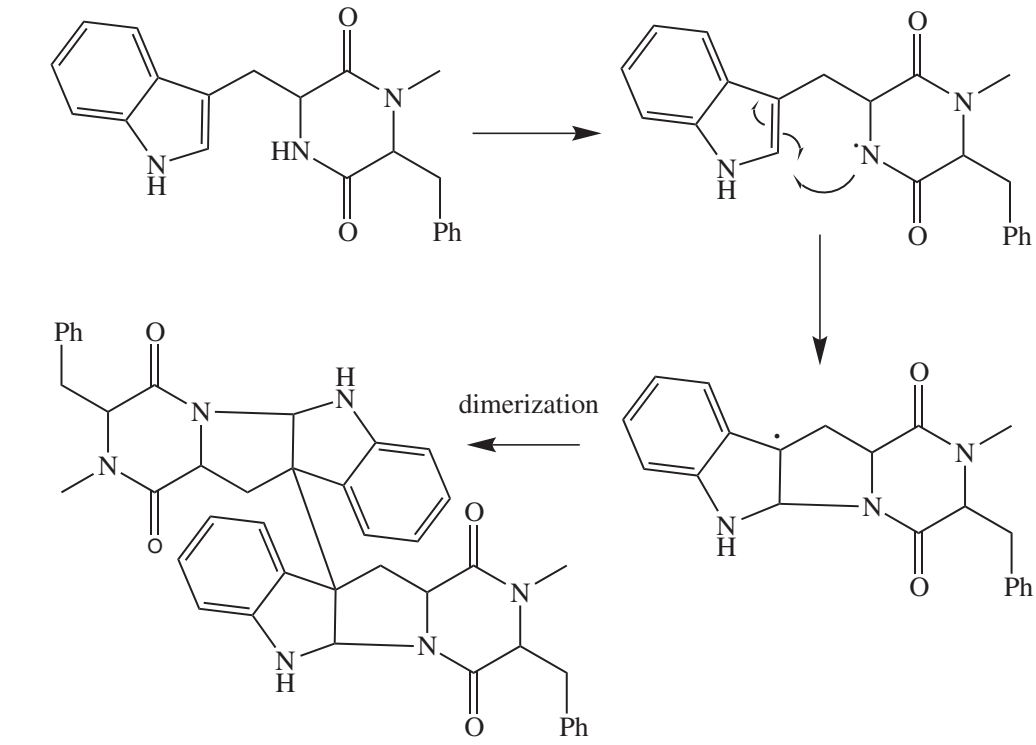

Fig. 4.41 Proposed mechanism formation of the alkaloid ditryptophenaline involving radical formation, cyclization, and subsequent dimerization of two substrate molecules catalyzed by cytochrome P450 DtpC

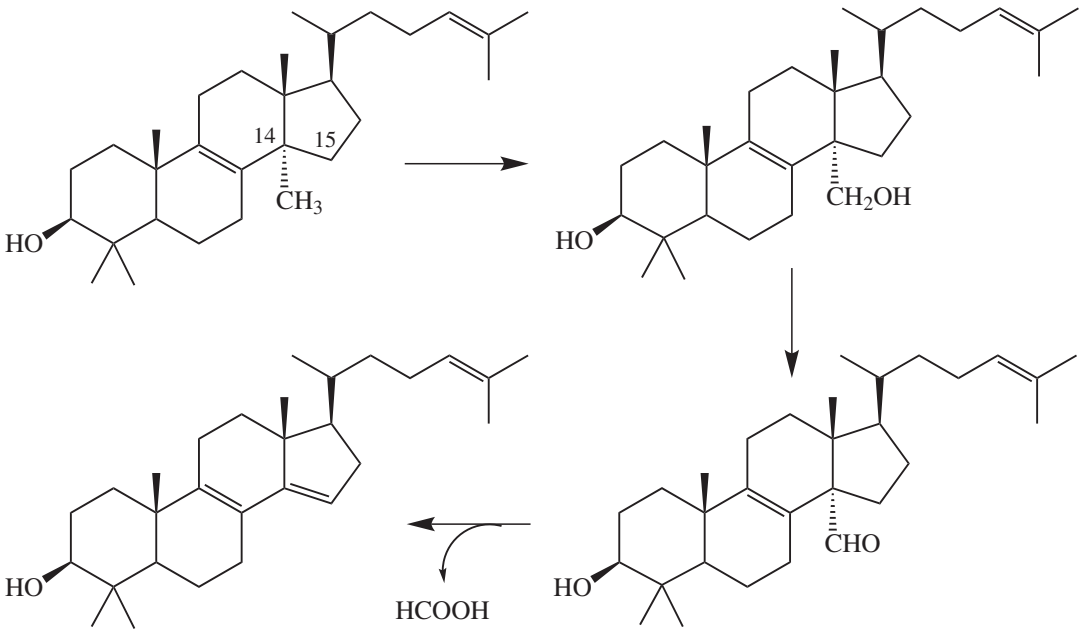

Fig. 4.42 The three oxidative steps in the CYP51-catalyzed conversion of lanosterol to the 14-demethylated sterol

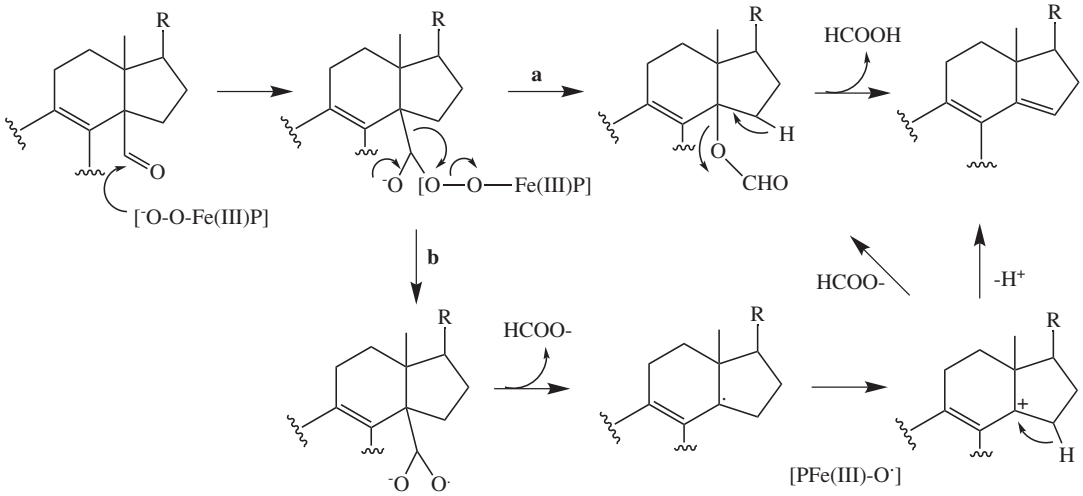

Fig. 4.43 Two alternative mechanisms for the C–C bond cleavage reaction that occurs in the third catalytic turnover of lanosterol by CYP51

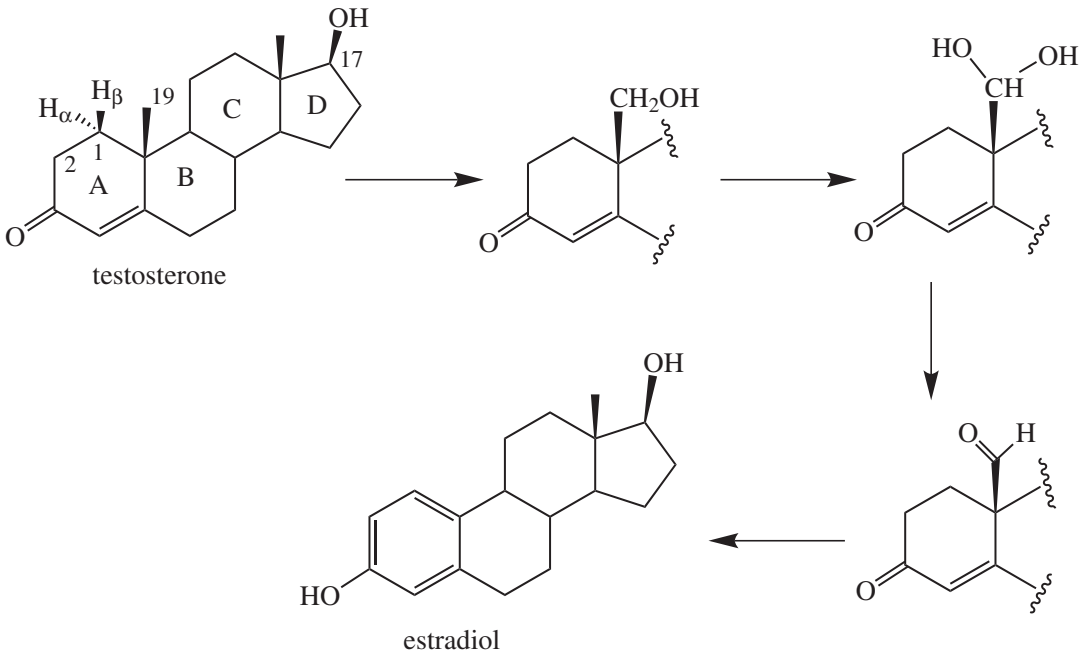

Fig. 4.44 The three overall reactions catalyzed by CYP19 (aromatase) that convert testosterone to 17β-estradiol

the 19-aldehyde group to give a peroxyhemiacetal (Fig. 4.45). Homolytic fragmentation of this peroxyhemiacetal generates an alkoxy radical that decays with loss of formic acid to a C10 radical. Compound II that is concomitantly formed in the reaction then abstracts the 1β-hydrogen to introduce a double bond. Ketone enolization finally converts the A-ring to the aromatic phenol found in estradiol, although enolization may occur prior to C–C bond cleavage. However, the CYP19A1-catalyzed oxidation of dihydrotestosterone, an analogue without the 4,5-double bond, produces 19-demethylated products with a 1,10-, 5,10-, or 9,10-double bond [258]. This result clearly indicates that enolization of the 3-keto function is not a prerequisite for the C–C bond-cleaving function

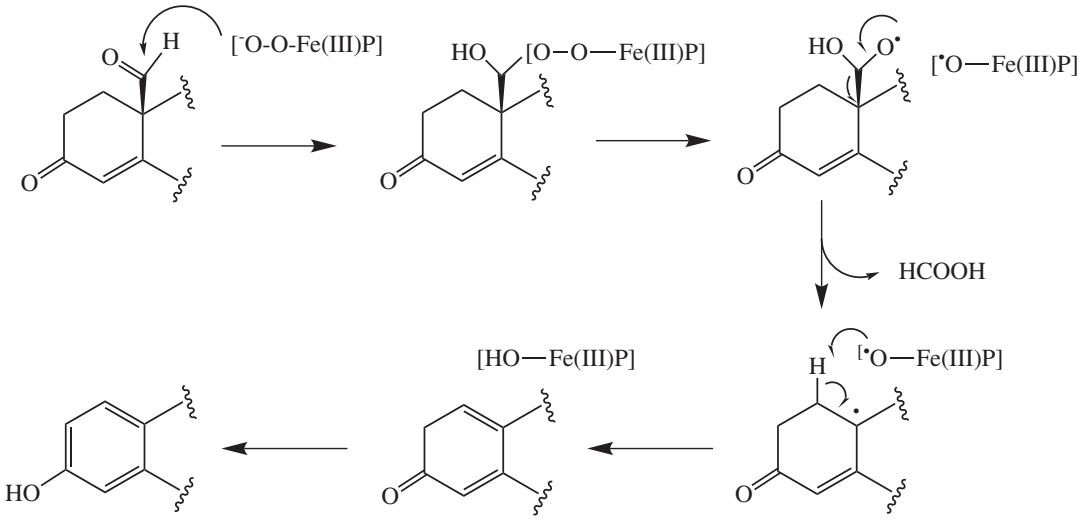

Fig. 4.45 The most commonly cited mechanism for the C–C bond cleavage reaction that occurs in the conversion of testosterone to 17β-estradiol by CYP19A1. An analogous sequence can be written in which the 3-keto group is first converted to the enol

of CYP19A1. The crystal structure of full-length human placental CYP19A1 demonstrates a tight binding site for the substrates and provides information on putative catalytic residues [259–261].

A DFT analysis of the C–C bond cleavage step catalyzed by CYP19 suggests that the above mechanism is not favored [262]. Specifically, the 1β-hydrogen abstraction in this sequence is calculated to have a high-energy barrier. The authors therefore proposed a mechanism in which the 3-enolized form of the 19-*gem*-diol undergoes 1β-hydrogen abstraction by a compound I ferryl species, producing first a C1 radical and then C1 cation, resulting in extrusion of formic acid and aromatization (Fig. 4.46). Subsequently, the authors revised their mechanism and returned to one initiated by addition of the ferric hydroperoxy anion to the 19-aldehyde, but with some subtleties in the subsequent steps [263]. These computational results remain hypothetical, but they emphasize that the details of the CYP19A1-catalyzed C–C fragmentation reaction are open to further definition. Some support for the computationally suggested mechanism is provided by resonance Raman studies of the hydrogen-bonding patterns for the CYP19A1 ferrous dioxygen intermediate complexed with either androstenedione or its 19-oxo derivative [264]. The studies indicate that in CYP19A1 there is a hydrogen bond to the terminal oxygen of the ferrous dioxygen intermediate with both substrates, a finding that suggests the same compound I intermediate will be generated in the C–C bond cleavage reaction as in the first hydroxylation step. The authors cite unpublished solvent isotope effect data that they claim are consistent with involvement of a compound I ferryl species in the C–C bond cleavage reaction.

4.7.1.3 Decarbonylations

CYP51 and CYP19 are examples of enzymes that break a C–C bond between a carbonyl group and an adjacent carbon, in each case with loss of the carbonyl function as formic acid, but other P450-catalyzed C–C bond-breaking reactions of carbonyl groups are known. One example is provided by housefly CYP4G, which oxidizes long-chain aldehydes to hydrocarbons with release of the aldehyde function as CO_2 rather than formaldehyde [265]:

$$CH_3(CH_2)_{15}CH_2CHO \rightarrow CH_3(CH_2)_{15}CH_3 + CO_2.$$

This reaction is closely related to the reaction catalyzed by another housefly enzyme, CYP6A1, for which it has been shown by deuterium labeling that the hydrogens at C2 and C3 are retained in the hydrocarbon when the aldehyde group (C1) is lost [266]. Most surprisingly, the labeling studies indi-

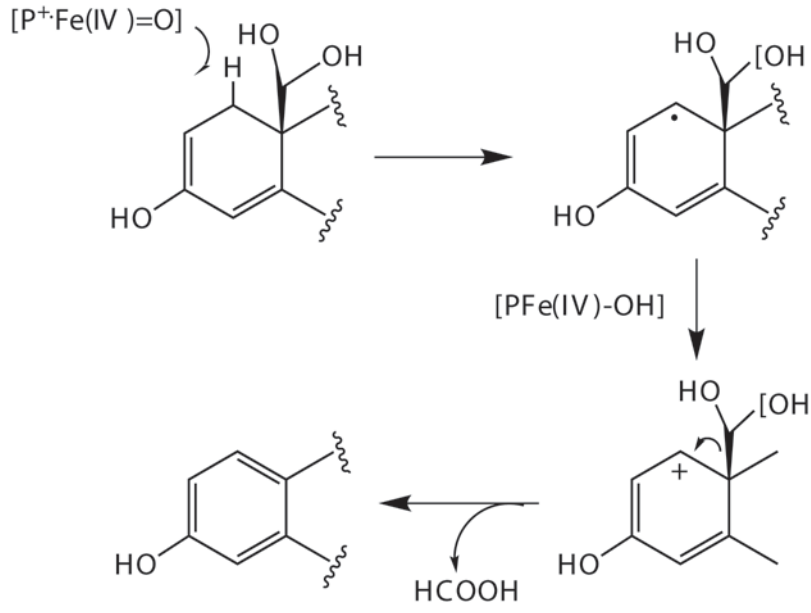

Fig. 4.46 An alternative mechanism suggested by computational studies for the C–C bond cleavage step in the oxidation of testosterone by CYP19A1

cated that the aldehyde hydrogen is transferred to the terminal carbon of the hydrocarbon product. A possible mechanism for this transformation postulates the addition of the ferric hydroperoxy anion to the aldehyde, followed by homolytic fragmentation to give formic acid and a carbon radical. The carbon radical then abstracts the hydrogen from the formate before it escapes from the active site, producing the hydrocarbon with retention of the original aldehyde hydrogen (Fig. 4.47). However, the authors reported that replacing NADPH and O_2 with H_2O_2, cumene hydroperoxide, or iodosobenzene supported enzymatic product formation for short periods. The mechanism in the figure is not consistent with these results, which led to the proposal of an unprecedented mechanism

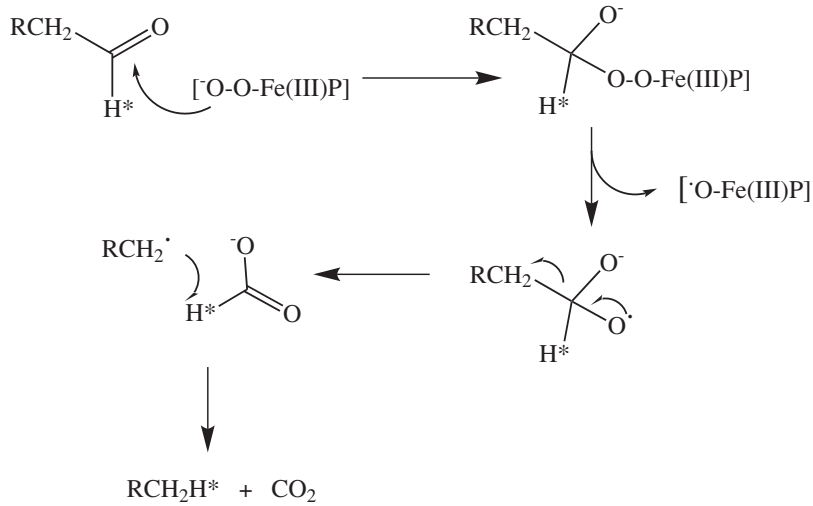

Fig. 4.47 Mechanism proposed for the decarbonylation of long-chain aldehydes by housefly cytochrome P450 enzymes. A surprising feature of the reaction is that the aldehyde hydrogen (*H**) is reportedly retained in the final hydrocarbon product

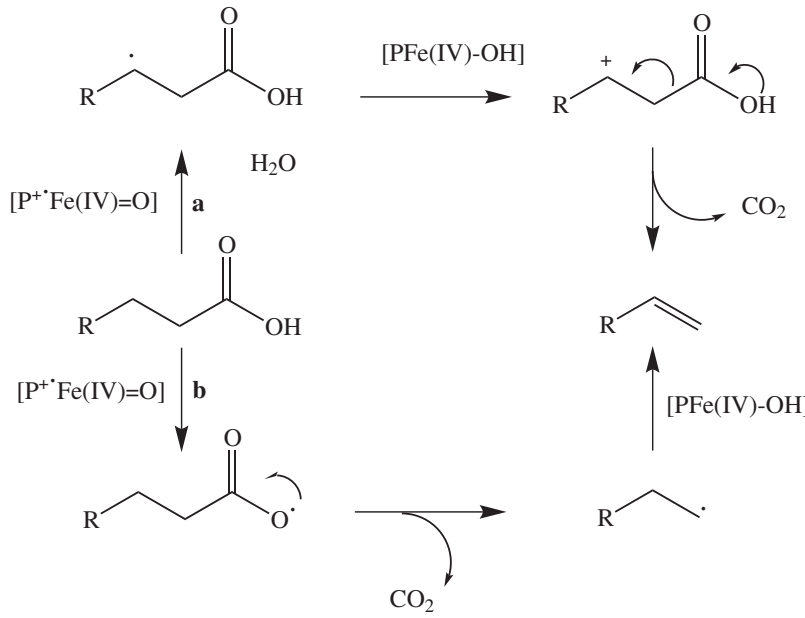

Fig. 4.48 Two mechanisms for the decarboxylation of fatty acids to terminal olefins catalyzed by CYP152L1, one involving β-carbon oxidation and the other carboxyl group oxidation. In mechanism *b,* the compound II intermediate abstracts the second hydrogen to generate the double bond

triggered by abstraction of an electron from the aldehyde carbonyl group [266]. Further work is clearly required to clarify the mechanism of this decarbonylation process, including confirmation of the anaerobic activity of the highly purified enzyme with surrogate oxidizing agents.

A variant of this transformation is catalyzed by CYP152L1 from the bacterium *Jeotgalicoccus* sp. 8546 [267] (Fig. 4.48). This H_2O_2-dependent P450 enzyme catalyzes the decarboxylation of fatty acids to give terminal olefins and carbon dioxide. Two fundamentally different mechanisms can be postulated for this transformation. Pathway *a* involves hydrogen abstraction from the carbon beta to the carboxyl group, oxidation of the resulting carbon radical to the cation by electron transfer to the compound II oxidizing species, and finally a chemically favored decarboxylation. This mechanism has precedent in the growing number of reactions in which a catalytically generated cation plays a role. Furthermore, the structure of the enzyme is quite similar to that of a homologue that catalyzes hydroxylation of the β-carbon. The alternative pathway *b* invokes one-electron oxidation of the carboxyl group, homolytic decarboxylation, and either a hydrogen abstraction from the β-carbon by the compound II species or oxidation of the primary radical to a cation followed by proton loss. These two alternatives are similar to those in the desaturation of hydrocarbons (Fig. 4.13). A limited precedent for P450-catalyzed carboxyl group oxidation is provided by the probable role of such an oxidation in the autocatalytic covalent attachment of the prosthetic heme group to the protein in some P450 enzymes [268].

4.7.2 Cleavage Between a Carbonyl and Hydroxyl Group

4.7.2.1 CYP17A1

CYP17A catalyzes both the 17α-hydroxylation of progesterone and subsequent cleavage of the C17–C20 bond to give androstenedione (Fig. 4.49). A similar transformation sequence accounts for the conversion of pregnenolone to dehydroepiandrosterone. The favored mechanism for this reaction involves formation of the hydroperoxy hemiacetal with the ferric hydroperoxy anion of the enzyme, followed by O–O bond homolysis, C–C bond homolysis, and finally recombination of the compound II equivalent with

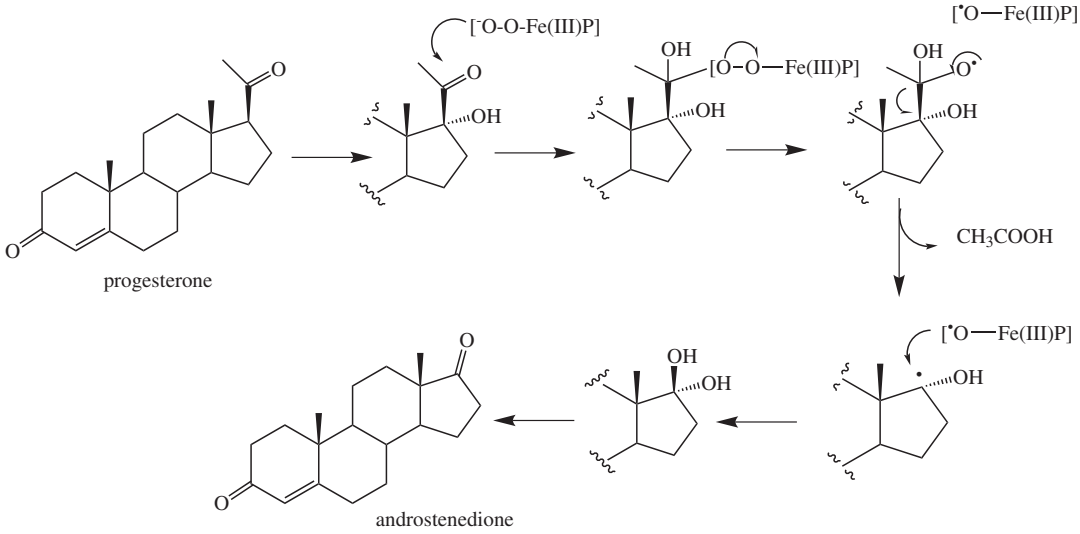

Fig. 4.49 Mechanism proposed for the C–C bond-breaking step in the oxidation of progesterone to androstenedione by CYP17A1

the C17 radical to give a *gem*-diol. Dehydration of this diol then produces the final product [253]. The same enzyme also catalyzes the formation of alternative minor products in a cytochrome b_5-dependent manner. The mechanism of this reaction is addressed in some detail in Chaps. 3 and 12 and is therefore not discussed further here.

4.7.2.2 Nabumetone

A nonsteroidal example of a C–C bond cleavage similar to that catalyzed by CYP17A1 is provided by the oxidation of nabumetone by CYP1A2. Nabumetone is an anti-inflammatory prodrug that is oxidatively converted to 6-methoxy-2-naphthylacetic acid (6-MNA), the physiologically active agent. Despite decades of clinical use, the mechanism of nabumetone bioactivation remained obscure until the recent demonstration that nabumetone is first hydroxylated to give 3-hydroxynabumetone. The CYP1A2-catalyzed C–C lysis of this hydroxyketone intermediate then yields an aldehyde, which in the third catalytic turnover of the enzyme is oxidized to the acid function found in 6-MNA (Fig. 4.50) [269, 270]. With the help of synthetic compounds, it has been established that the C–C bond cleavage step occurs from the 3-hydroxyketone and not from the parent ketone or the 2,3-diol, which can be formed biologically by reduction of the ketone [269]. Furthermore, although nabumetone 3-hydroxylation was supported when cumene hydroperoxide was employed instead of NADPH and cytochrome P450 reductase, neither nabumetone nor 3-hydroxynabumetone was converted to 6-MNA under these conditions. As the enzyme retained the ability to catalyze 3-hydroxylation with cumene hydroperoxide, but not C–C bond cleavage, it appears that the C–C bond cleavage involves addition of the ferric hydroperoxy anion to the carbonyl group of nabumetone.

4.7.2.3 CYP24A1 (Vitamin D_3 Oxidation)

Vitamin D_3 and its 25-hydroxy derivative undergo a side-chain cleavage reaction catalyzed by CYP24A1 [271]. In this side-chain cleavage process, CYP24A1 catalyzes 24-hydroxylation of the side chain and then a second hydroxylation to generate the 24-ketone. A third hydroxylation produces the 23-hydroxy-24-ketone that actually undergoes the C–C bond cleavage reaction. There is some uncertainty in the literature on the nature of the product formed by CYP24A1 in the C–C bond cleavage step [272–275]. The current literature tends to favor formation of the truncated 23-alcohol, which is then sequentially oxidized to the 23-aldehyde and 23-acid by the same enzyme (Fig. 4.51) [271]. However, a mechanism for direct conversion of the 23-hydroxy-24-keto structure to the

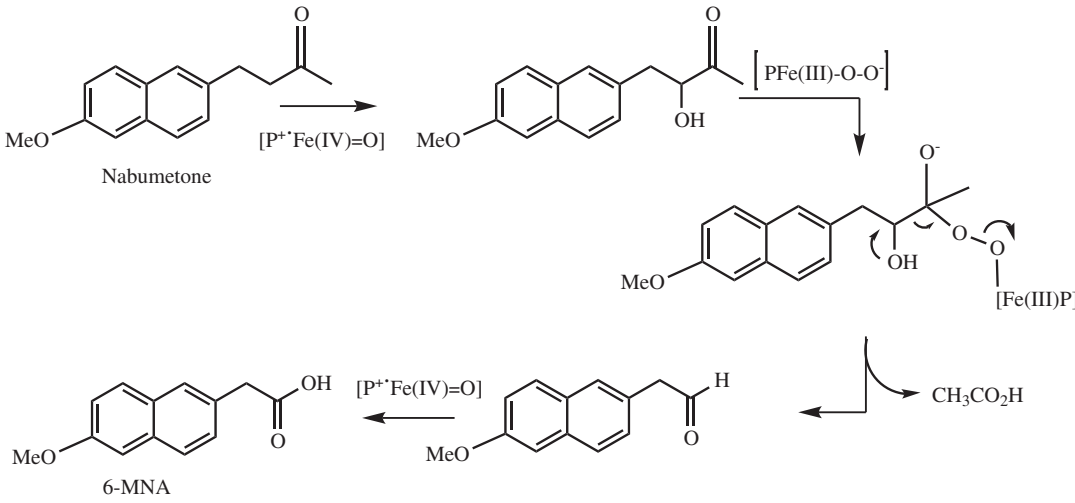

Fig. 4.50 Three-step oxidative sequence for oxidation of the prodrug nabumetone to the physiologically active metabolite 6-MNA by CYP1A2

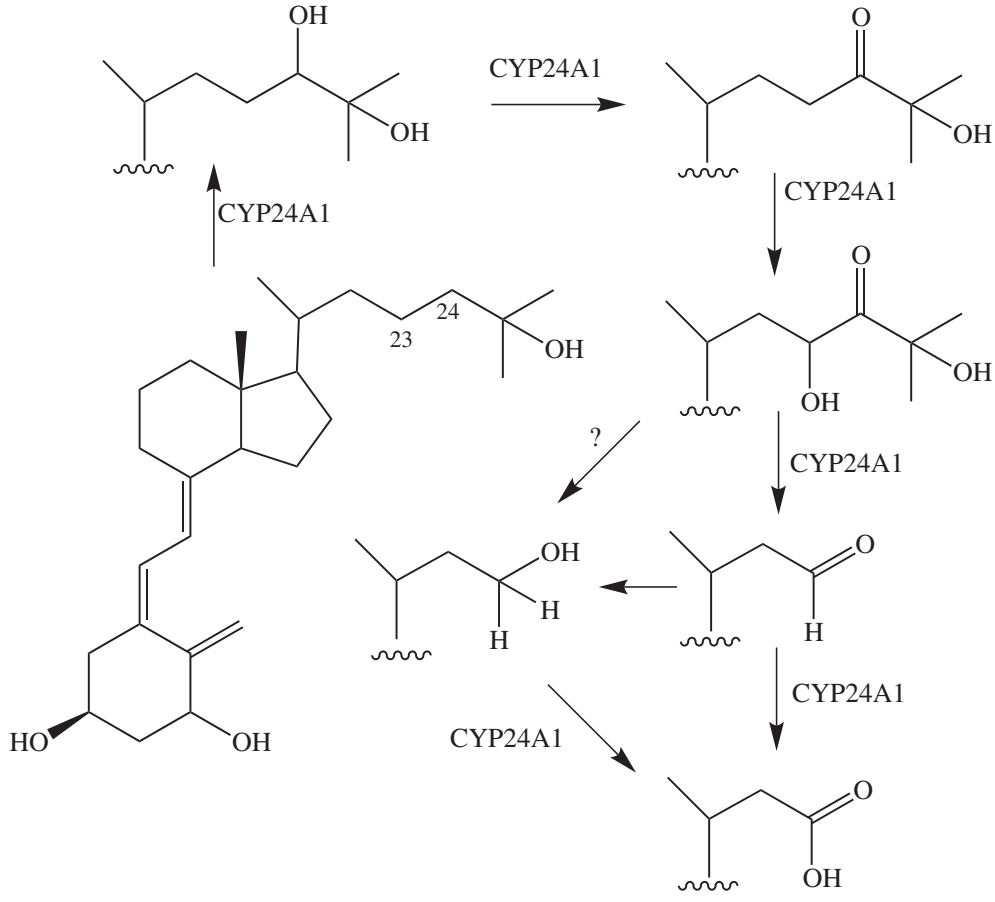

Fig. 4.51 Multistep CYP24A1-catalyzed oxidation of 25-hydroxyvitamin D_3, including a C–C bond cleavage step, to the metabolite 24,25,26,27-tetranor-23-OH-D_3. The figure emphasizes the probable role of the 23-alcohol as a side product rather than as an obligatory intermediate in the side-chain cleavage sequence

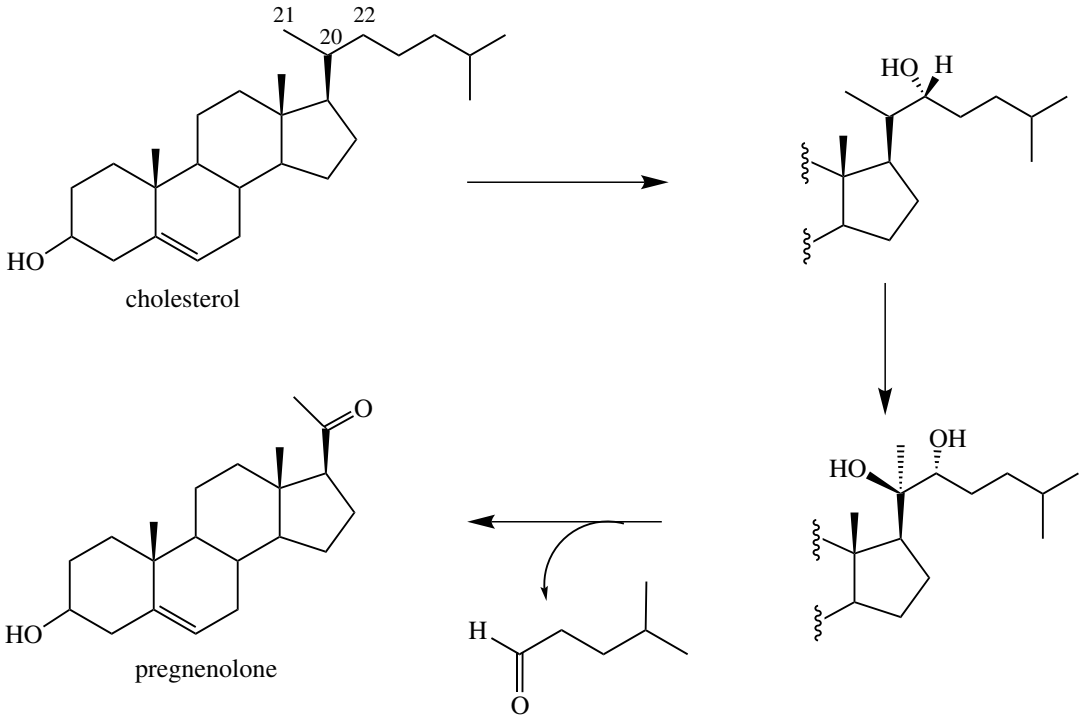

Fig. 4.52 The three-step side-chain cleavage of cholesterol to give pregnenolone catalyzed by CYP11A1

truncated 23-alcohol is difficult to reconcile with any P450 mechanism for which there is credible precedent. Mechanistic analysis suggests that the initial product should be the 23-aldehyde. As the 23-aldehyde is known to be unstable towards disproportionation that produces the 23-alcohol and 23-acid, it is most likely that the experimentally documented formation of the 23-alcohol is the result of a side reaction. It has been shown that the 23-alcohol can be oxidized by CYP24A1 to the aldehyde and acid, but this does not require that the 23-alcohol occur as an intermediate in the sequence that normally leads to the 23-acid. If the aldehyde is the immediate product, the mechanism would be similar to that postulated for the oxidation of nabumetone (Fig. 4.50).

4.7.3 Cleavage Between Two Hydroxyl Groups

4.7.3.1 CYP11A1 (Sterol Side-Chain Cleavage)

The first step in the synthesis of sterol hormones from cholesterol is removal of the cholesterol side chain by CYP11A1 to give pregnenolone and 4-methylpentanal (Fig. 4.52). The two initial steps are conventional hydroxylations by the compound I ferryl species [276], first to give the 22(*R*)-hydroxylated sterol and then 20(*R*), 22(*R*)-dihydroxycholesterol. Subsequent cleavage of the C–C bond between the hydroxylated side-chain carbons produces pregnenolone with concomitant elimination of the rest of the side chain as 4-methylpentanal [277, 278]. In view of all the other sterol C–C bond-breaking reactions that involve a carbonyl group, it is important to note that the 22(*S*)-hydrogen of the side chain is retained in the 4-methylpentanal, precluding oxidation of the 22-alcohol to a ketone prior to bond scission [277]. The C–C bond cleavage must therefore occur from the diol. One possibility is that the compound I ferryl oxygen abstracts a hydrogen atom from one of the hydroxyl groups, producing an alkoxy radical that fragments into 4-methylpentanal and a sterol with a C20 radical. Electron transfer from the radical to the enzyme then would produce the ketone, as would recombination to deliver a hydroxyl to the carbon followed by a dehydration reaction (Fig. 4.53, path

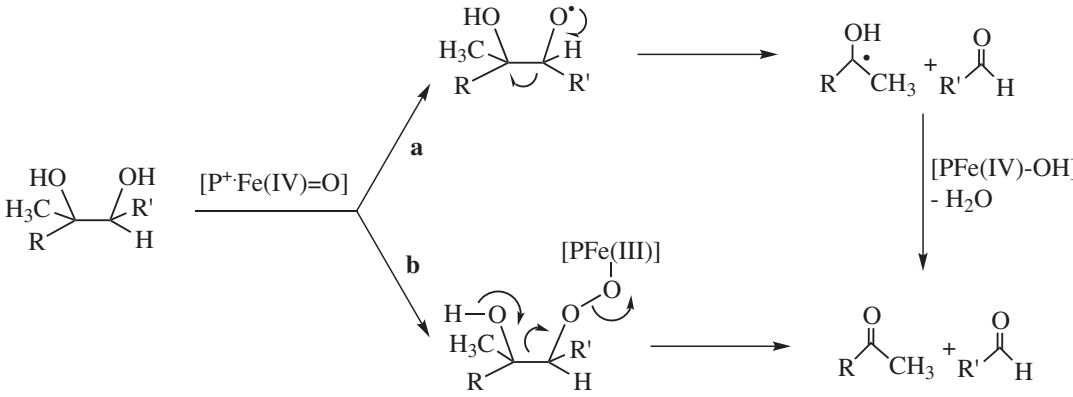

Fig. 4.53 Two alternative mechanisms for the C–C bond cleavage between two alcohol groups that occurs in the reaction mediated by CYP11A1

a). However, a second possible mechanism postulates that the C22-hydroxyl adds to the compound I ferryl oxygen, forming a hydroperoxy structure that subsequently fragments to the observed products (Fig. 4.53, path b). Determination of the crystal structure of CYP11A1 complexed with reaction intermediates by two laboratories [279, 280] led one of them to suggest that the more dynamic nature of the C22- than C20-hydroxyl supports the mechanism invoking addition of the C22-hydroxyl to the compound I ferryl species [279]. However, although chloride ion adds to the compound I ferryl oxygen in enzymes like chloroperoxidase to form an Fe–O–Cl complex, there is little independent evidence that a hydroxyl group can similarly add to generate a ferric hydroperoxo intermediate.

4.7.3.2 CYP107H1

$P450_{BioI}$, formally classified as CYP107H1, catalyzes the cleavage of a diol in the middle of a fatty acid chain to give two aldehyde fragments, although it actually utilizes as a substrate a fatty acid covalently attached to an acyl carrier protein (ACP) [281, 282]. The crystal structure of the complex of the protein with the ACP–fatty acid substrate shows that it binds in a U-shaped conformation that places the C7 and C8 carbons of the fatty acid chain directly above the heme iron atom, explaining the regiospecificity of the reaction. Earlier studies with free fatty acids, which are oxidized in low yield, showed that the best substrates had a *threo*-7,8-diol substitution, whereas the 7-oxo, 8-oxo-, 8-hydroxy-, or erythro-7,8-diol were not acceptable as substrates [283]. The enzyme thus catalyzes two conventional hydroxylation reactions on the same face of the fatty acid chain before cleaving the C–C bond at the 7,8-diol stage (Fig. 4.54). The mechanism of this C–C bond cleavage has not been more precisely defined, but it is reminiscent of the side-chain cleavage reaction catalyzed by CYP11A1 in the conversion of cholesterol to pregnenolone (Fig. 4.52).

4.7.4 Other C–C Bond-Cleaving Reactions

4.7.4.1 Fumagillin Biosynthesis

A highly unusual transformation occurs in the biosynthesis of fumagillin by *Aspergillus fumigatus* [284]. As shown in Fig. 4.55, the bicyclic terpene skeleton of β-*trans*-bergamotene is first hydroxylated by a cytochrome P450 enzyme, termed Fma-P450, at a bridgehead position to give the corresponding tertiary alcohol. In a second step, the same P450 enzyme catalyzes carbon–carbon cleavage with the concomitant formation of epoxide and ketone functions. In the final step of the sequence, the same P450 enzyme promotes epoxidation of the exocyclic double bond. The first and third steps in this sequence are conventional cytochrome P450 reactions, but

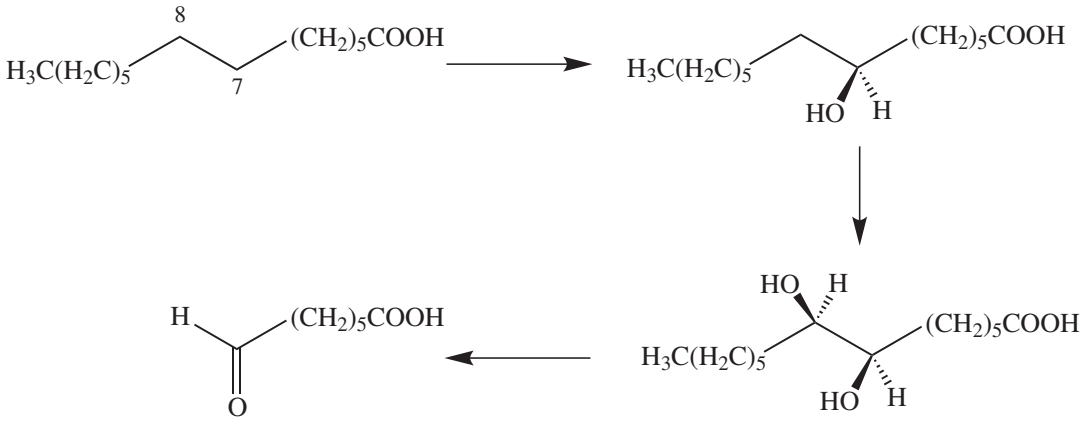

Fig. 4.54 The two hydroxylations and C–C bond cleavage reaction catalyzed by the enzyme BioI

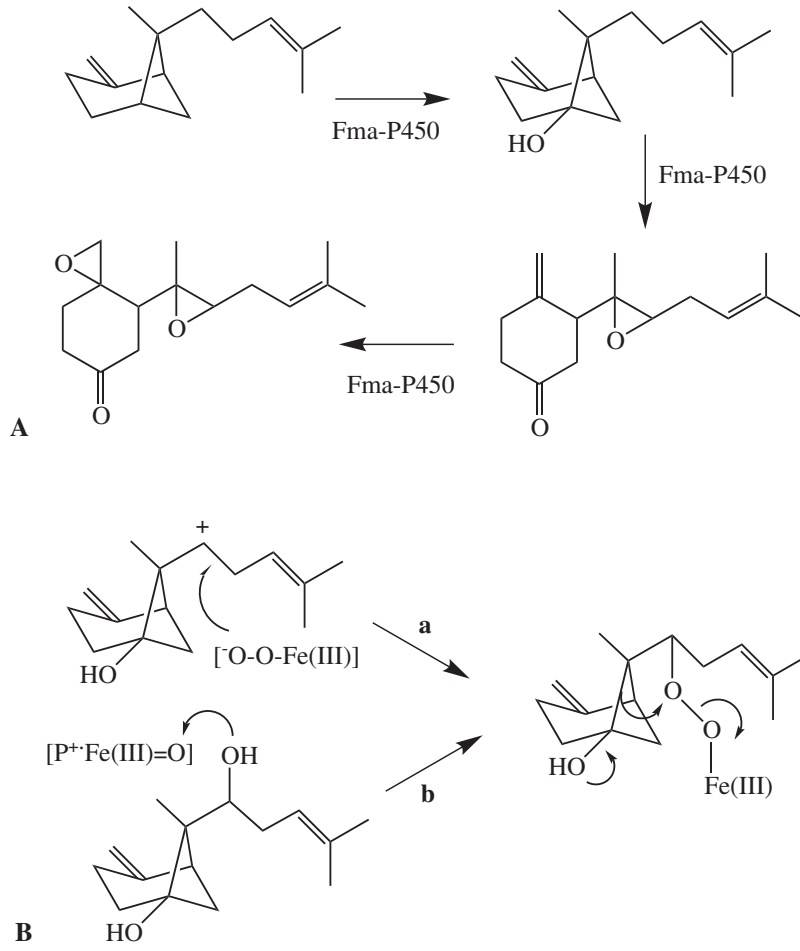

Fig. 4.55 Fma, a cytochrome P450 enzyme, catalyzes an unusual C–C bond cleavage reaction that directly generates an epoxide function. This reaction can be rationalized by formation of a peroxoiron adduct with the enzyme by either of the two mechanisms shown in part **b** of the figure

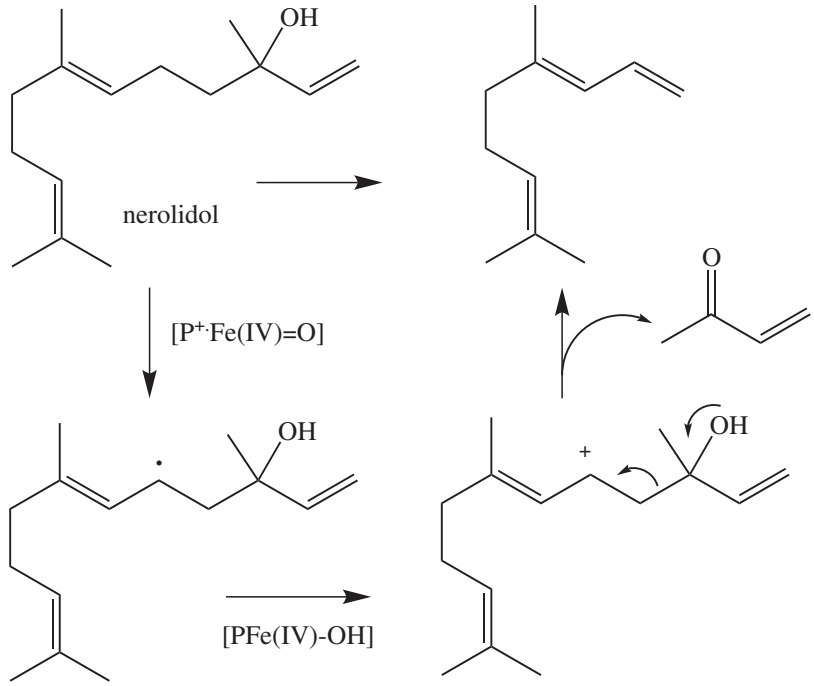

Fig. 4.56 C–C bond cleavage reaction in the CYP82G1-catalyzed formation of terpene hydrocarbons from precursor alcohols

the intervening carbon–carbon bond-cleaving reaction is unexpectedly complex. To explain this transformation, the authors have proposed that hydrogen abstraction from the side chain to give a carbon radical followed by electron transfer to compound II generates a cation (Fig. 4.55b, path a). This cation is then trapped by the ferric peroxy anion produced by a further catalytic turnover of the enzyme, with the resulting alkylperoxyiron intermediate undergoing fragmentation to directly form the keto-epoxide product. A shortcoming of this otherwise-clever mechanism is that the cation that is formed in one catalytic cycle must remain intact while the enzyme undergoes a second activation of molecular oxygen. An alternative would be for the enzyme to perform a conventional hydroxylation (Fig. 4.55b, path b), with the resulting stable alcohol then reacting in a second catalytic cycle with compound I of the enzyme to produce the same alkylperoxo species as in path a. Alternative mechanisms are possible, however, including C–C bond cleavage by the radical that precedes the cation in path a, introducing a double bond into the side chain, and placing the radical on the carbon adjacent to the hydroxyl group of the cyclohexyl ring. Electron transfer to compound II would then generate the ketone and a subsequent catalytic turnover would epoxidize the side-chain double bond to give the final product.

4.7.4.2 CYP82G1 (Terpene Hydrocarbon Synthesis)

The damage caused by herbivore attack on *Arabidopsis* results in the emission of two terpene defense molecules, (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) and (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT). These molecules are generated by a single cytochrome P450 enzyme, CYP2G1, from tertiary alcohol terpene precursors, with nerolidol as the precursor for the second product (Fig. 4.56) [285]. CYP2G1 has a very narrow specificity for these substrates. Although the mechanism was not further investigated, the most plausible mechanism would appear to involve allylic hydrogen abstraction by

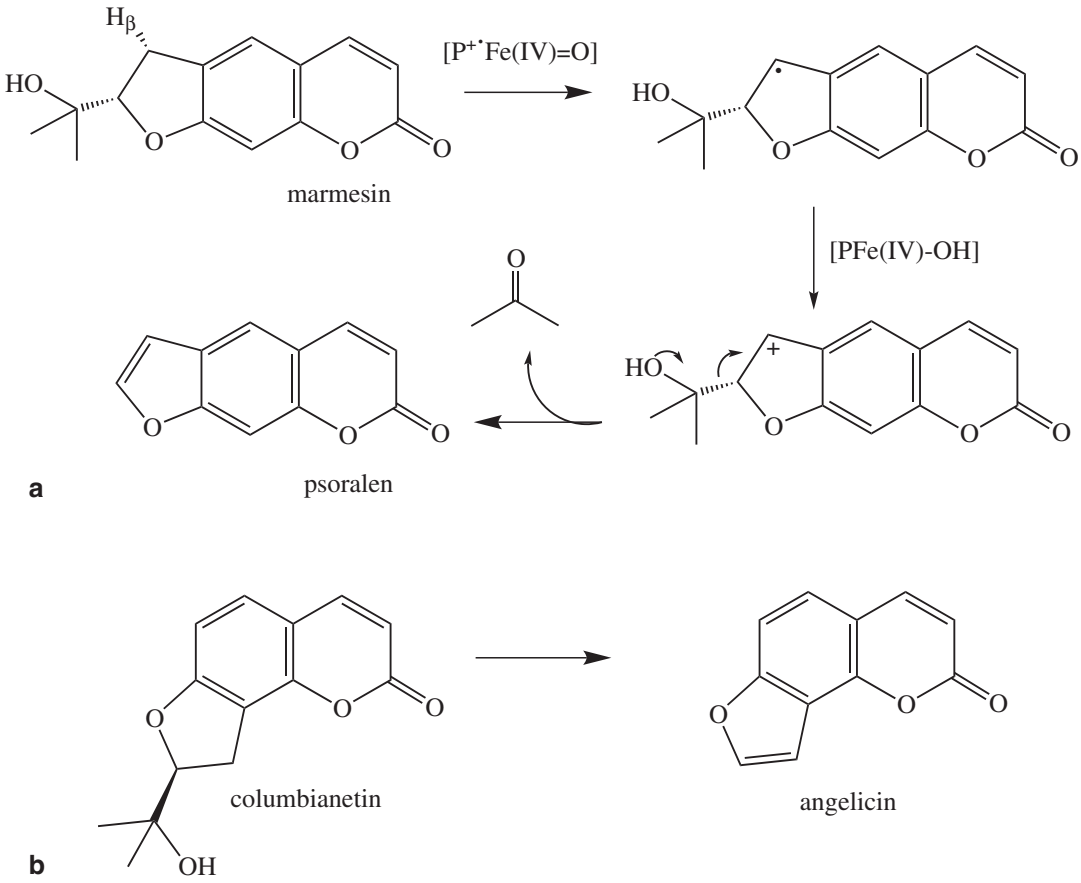

Fig. 4.57 C–C bond cleavage reactions in the formation of psoralen from marmesin (**a**), and angelicin from columbianetin (**b**)

compound II followed by electron transfer to the enzyme to generate the allylic cation. Hydroxyl-driven fragmentation then produces DMNT from nerolidol together with 3-buten-2-one. The conjugated ketone product was not detected, but this might be due to the fact that it is a highly reactive Michael acceptor and is likely to be trapped by nucleophiles in the incubations.

4.7.4.3 Furanocoumarin Biosynthesis

The conversion of marmesin to psoralen and acetone is catalyzed by CYP71AJ1 from the plant *Ammi majus* (Fig. 4.57a) [286, 287]. Deuterium labeling studies established an elimination stereochemistry in which the β-hydrogen was lost. Three orthologues of this enzyme, CYP71AJ2 from *Apium graveolens* and CYP71AJ3 and CYP71AJ4 from *Pastinaca sativa,* have been identified and expressed in yeast cells. Two of these enzymes, CYP71AJ2 and CTO71AJ3, have psoralen synthase activity, but CYP71AJ4 only catalyzes the conversion of columbianetin to angelicin (Fig. 4.57b) [288]. A free radical fragmentation mechanism was proposed for the formation of psoralen, but a more attractive mechanism is shown in Fig. 4.57a. In this mechanism, the fragmentation does not occur at the stage of the carbon radical, but rather after the radical is oxidized to a cation by electron transfer to the enzyme. This mechanism closely resembles that proposed for CYP82G1 above. Furthermore, a very similar mechanism can be written for the conversion of columbianetin to angelicin. Support for this mechanism is provided by the obser-

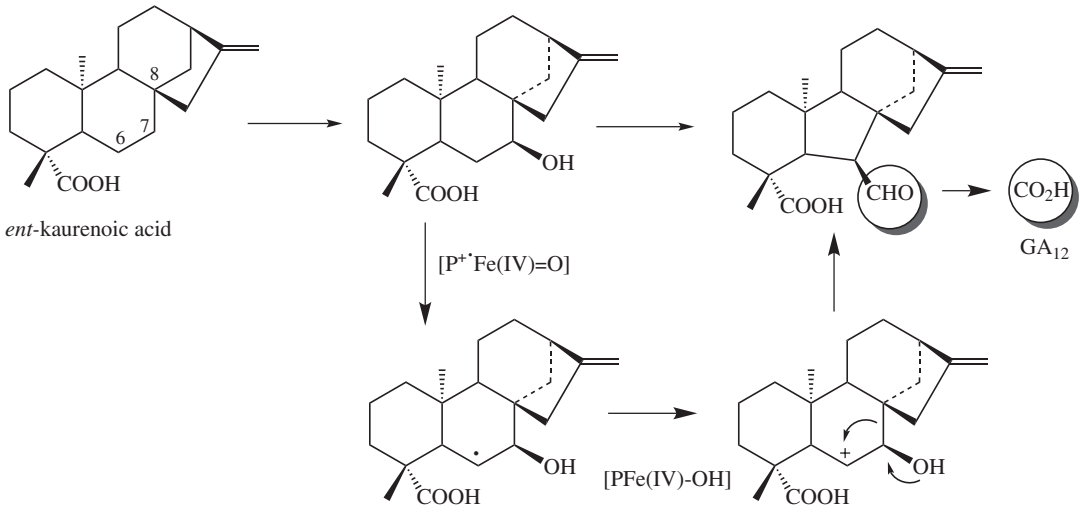

Fig. 4.58 Mechanism that rationalizes the ring contraction reaction in the conversion of *ent*-kaurenoic acid to a precursor of GA_{12} in the gibberellin biosynthetic pathway. The *circle* highlights the oxidation of the aldehyde to the acid that is required in the final step of GA_{12} synthesis

vation that stereospecific *syn*-deuterium substitution at the 3′-carbon that is oxidized in columbianetin causes a metabolic switch that produces 3′-hydroxylated columbianetin rather than leading to elimination of the side chain. This result specifically excludes reaction mechanisms that are initiated by oxidation of the hydroxyl group in the side chain.

4.7.4.4 CYP88A (Gibberellin Biosynthesis)

A key transformation in the biosynthesis of gibberellin is the conversion of *ent*-kaurenoic acid to GA_{12}, and the critical step in this transformation is the six- to five-membered ring contraction that generates an aldehyde that is subsequently oxidized to the acid function of GA_{12} (Fig. 4.58). The sequence of hydroxylation, ring contraction, and oxidation of the aldehyde to the acid is catalyzed by CYP88A from *Arabidopsis thaliana* [289], barley [289], and *Gibberella fujikuroi* [290]. In the case of the *Gibberella* studies, the 6,7-diol was isolated but was not converted to GA_{12} by the enzyme, suggesting that the diol was a side product rather than a precursor of GA_{12}. The detailed mechanism of the ring contraction reaction remains undefined, but it is likely to involve hydrogen abstraction to give the carbon radical adjacent to the hydroxyl group, electron transfer to the enzyme to produce the cation, and finally ring contraction with concomitant formation of the aldehyde, as illustrated in Fig. 4.58.

4.7.4.5 Pentalenolactone Biosynthesis

Cytochrome P450 enzymes in *Streptomyces exfoliates* and *Streptomyces arenae,* labeled as PenN and PenM, catalyzed a methyl migration on a saturated ring (Fig. 4.59) [291]. As shown in the figure, this rearrangement involves stereospecific removal of the hydrogen *trans* to the migrating methyl to give the carbon radical, oxidation of the radical to the cation by electron transfer to the enzyme, migration of the methyl to the cationic site, and deprotonation to product the final double bond.

4.8 Perspectives

Direct observation and characterization of the cytochrome P450 compound I ferryl species over the past few years has affirmed its role as the key oxidizing species in cytochrome P450-catalyzed hydroxylations. If the ferric hydroperoxide pre-

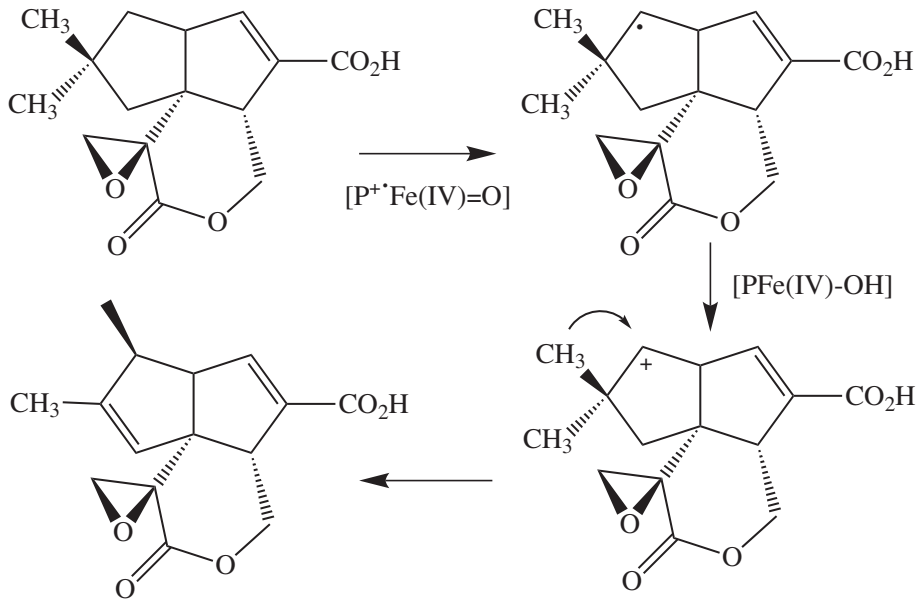

Fig. 4.59 Methyl migration in the final step of pentalenolactone biosynthesis catalyzed by *Streptomyces* cytochrome P450 enzymes

cursor of compound I (Fig. 4.1g) participates in substrate oxidations, its role is minor and is confined to easily oxidizable centers, such as nitrogen and sulfur atoms. In contrast, the evidence for involvement of the ferric hydroperoxy anion (Fig. 4.1f) as a nucleophilic oxidant, particularly one involved in carbonyl C–C bond-cleaving reactions, is now well established. However, its role in some reactions, such as the aromatization catalyzed by CYP19, is being challenged as increasingly refined spectroscopic and biochemical tools are used to probe the mechanism. It is to be expected that in the next few years the mechanisms of P450 reactions will be defined at a much higher "resolution" than previously, and that some mechanisms thought to be settled will require revision.

The growth in the studies of nonmammalian cytochrome P450 systems, particularly those of plants and microbes, has unearthed a rich and unforeseen complexity of cytochrome P450-catalyzed transformations. The breadth of cytochrome P450 catalysis will surely continue to grow as these still relatively unexplored biological domains reveal the diversity of aims to which cytochrome P450 enzymes have been adapted by evolution. Recent work in this area suggests that the role of cations in cytochrome P450-catalyzed transformations may be greater than previously envisioned, particularly in reactions that result in cleavage or rearrangement of C–C bonds. Although radical mechanisms can be written for some of these reactions, in many instances the products are more reasonably explained by sequential oxidation of a C–H bond to a carbon radical and then a cation.

We can look forward in the near future to a deeper molecular understanding of the mechanisms of cytochrome P450 enzymes, of the interactions of substrates with P450 proteins that influence the catalytic outcome, and of the range of transformations that are possible with the versatile catalytic machinery of these enzymes.

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Inhibition of Cytochrome P450 Enzymes

5

Maria Almira Correia and Paul. F. Hollenberg

5.1 Introduction

Inhibition of cytochrome P450 (P450, CYP) function (see Chaps. 3 and 4) may be brought about directly or indirectly. The steps in the P450 catalytic cycle particularly vulnerable to direct chemical inhibition include substrate binding to the ferric-P450 protein, molecular oxygen binding to the ferrous-P450, and subsequent insertion of the oxygen atom into the substrate. Direct functional inhibition, in principle, can also occur following posttranslational modifications of the P450 protein surface by oxidants, alkylating, nitrosating, or acylating agents that disrupt critical interactions with its redox partners, cytochrome P450 oxidoreductase (CPR), and/or cytochrome b_5 (b_5) [1, 2]. Selective antibodies targeted against P450 epitopes in these functionally relevant surface regions similarly disrupt P450 function and are valuable diagnostic probes [3–6]. On the other hand, indirect acting inhibitors may target other steps in the P450 catalytic cycle, such as the *sine qua non* CPR electron donation step, either through diversion of its electron supply away from the P450 hemoprotein [7–9] or by inactivating the CPR flavoprotein itself, i.e., with diphenyleneiodonium [10, 11]. Consistent with its unique role in the P450 catalytic cycle, conditional deletion of the CPR gene in vivo also results in P450 functional inhibition [12, 13]. Furthermore, P450 functional inhibition can also be elicited by agents that either impair protein or heme synthesis or accelerate protein or heme degradation (i.e., metals such as Co^{+2}), and thus effectively reduce P450 hemoprotein content [14]. Thus, although various modes of P450 inhibition exist and can be effectively exploited experimentally, physiologically, and/or therapeutically, only direct acting P450 chemical inhibitors will be discussed in this chapter.

Such direct acting P450 inhibitors can be classified into three mechanistically distinct groups: Agents that form (a) reversible complexes, (b) quasi-irreversible complexes with the heme-iron atom, and (c) "dead-end" complexes through irreversible interaction with the P450 protein or the heme moiety, or accelerated degradation and/or oxidative fragmentation of the prosthetic heme [15–33]. Reversible competitive or noncompetitive inhibitors are generally thought to interfere in the P450 catalytic cycle prior to the actual oxidative event. On the other hand, agents that act during or subsequent to the oxygen transfer step are generally considered to be irreversible or quasi-irreversible inhibitors. Indeed, because the manifestation of their intrinsic irreversible or quasi-irreversible inhibitory potential requires P450 catalytic turnover, such agents are often aptly classified as mechanism-based (or suicide) inactivators [21–30]. Extensive lists of

M. A. Correia (✉)
Department of Cellular and Molecular Pharmacology, University of California, 600 16th Street, N572, San Francisco, CA 94158-2517, USA
e-mail: almira.correia@ucsf.edu

P. F. Hollenberg
Department of Pharmacology, University of Michigan, Ann Arbor, MI 48109-5632, USA

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P450 inhibitors are available elsewhere [34–36], and Chap. 9 also covers inhibitors of individual P450 enzymes. This chapter focuses largely on the mechanisms of reversible P450 inhibition as well as the mechanisms of P450 inactivation and agents that function as mechanism-based inactivators. Despite their irrefutable practical relevance to clinical therapeutics, the mechanisms of reversible competitive and noncompetitive inhibitors, being relatively straightforward, are discussed more concisely.

5.2 Reversible Inhibitors

Agents that compete with substrates for the occupancy of the P450 active site through: (a) binding to hydrophobic regions of the active site, (b) coordination to the prosthetic heme-iron atom, or (c) specific hydrogen bonding or ionic interactions with active site residues are considered reversible inhibitors [18–25]. In the first case, the inhibitor simply competes for binding to the lipophilic domains of the active site, resulting in the type of inhibition often observed when two substrates compete for oxidation by a single P450 isoform. The mutual in vitro and in vivo inhibition of benzene and toluene metabolism is the simplest example of such a direct competitive interaction [37]. Such inhibition is optimally manifested upon prolonged residence of the inhibitory agent within the P450 active site due to its tight binding coupled with its poor catalytic recognition as a substrate. This is illustrated by the inhibition of CYP1A2-dependent caffeine or theophylline N-demethylation or of CYP19 -mediated estrogen synthesis by α-naphthoflavone (K_I 0.01 μM) [36]. Although usually not quite as effective as reversible inhibitors that interact with the P450 heme iron or irreversible P450 inactivators, nonetheless such interactions are responsible for eliciting not only relevant metabolic alterations but also many clinically significant drug–drug interactions (DDIs) [18, 19].

5.2.1 Coordination to the P450 Ferric Heme Iron

Amino-side chains or nitrogenous heterocyclic moieties in some substrates [20, 24, 25] can either directly bind tightly to the sixth coordination site of the pentacoordinated prosthetic P450 heme-iron atom or displace an existing weaker ligand such as water from a P450 hexacoordinated state (Fig. 5.1a, b) [23–25]. The ensuing P450 heme-

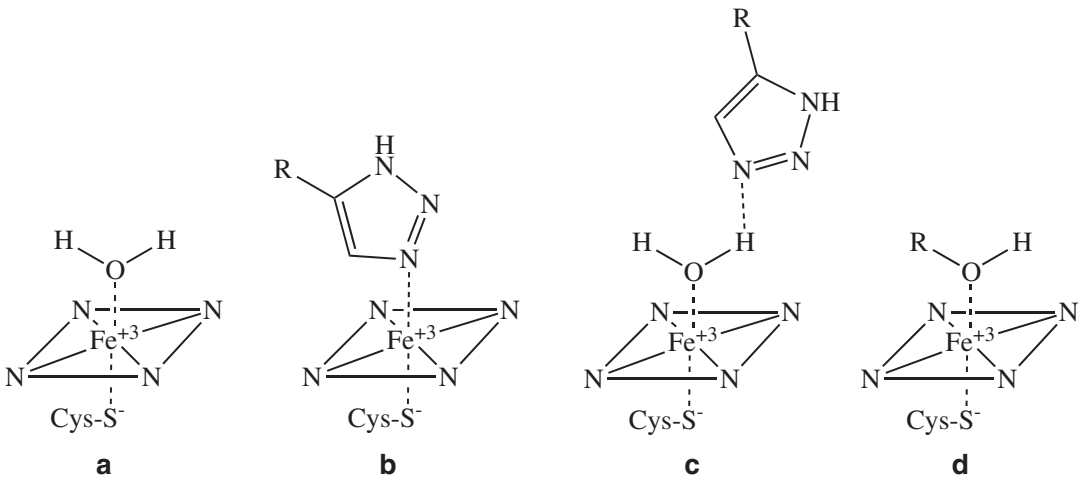


Fig. 5.1 P450 ferric-heme interactions at the sixth axial coordination site with various ligands. **a** Resting state with an active site water molecule bound; **b** Triazole nitrogen coordination associated with the spectroscopic type II signature; **c** "Pseudo" or type II-like binding via the axially coordinated water, associated with a spectral signature that resembles "reverse type I-binding"; **d** Coordination with the oxygen atom in alcohols, associated with a spectroscopic "reverse type I-binding"

iron-liganded complexes exhibit a shift of the iron from the high- to the low-spin state, with a characteristic spectroscopic signature: A "type II"binding spectrum with a Soret maximum at 425–435 nm and a trough at 390–405 nm [38–41]. This spin state change also alters the intrinsic P450 redox potential so as to impair its reduction by CPR (see Chap. 2) [41, 42]. This impaired reduction potential, just as much as the physical occupation of the sixth coordination site, accounts for the inhibition associated with the binding of strong heme-iron ligands. However, it is to be noted that not all substrates that exhibit "type II-like spectra" are necessarily P450 inhibitors and may actually be quite productive substrates depending on whether they are hydrogen-bonded to its hexacoordinated water ligand rather than directly hexacoordinated to the P450 prosthetic heme iron (Fig. 5.1c). On the other hand, model studies of synthetic iron porphyrin binding to various dialkylnitrosamines, reveal through electron paramagnetic resonance (EPR) spectroscopy the formation of isolable hexacoordinated low-spin and pentacoordinated high-spin ferric-porphyrin complexes. Single crystal X-ray crystallography of these complexes indicates that all these nitrosamines bind to the ferric iron atom via an n^1-O binding mode [43], thereby suggesting that similar complexes with the ferric P450 species are in principle also plausible. Furthermore, although per se not metabolically reactive, such complexes may acquire reactivity on reduction to the ferrous species.

The preferential, albeit weak binding of cyanide and other ionic ligands to the ferric P450 species [44, 45] has been discussed in detail previously [24]. It is to be underscored that the negatively charged cyanide ion favors the neutral ferric P450 species over the more negatively charged ferrous species. This preference may also account for its stronger binding of ferric myoglobin over the ferric P450 species, as the thiolate ligation of the latter enriches the electron density around the iron atom, making it considerably more negatively charged than does the imidazole ligand of ferric myoglobin [46]. The lipophilic nature of the P450 active site is an additional deterrent to its interactions with ionic ligands [47].

Nitric oxide ($^{\bullet}$NO), an intracellular signaling molecule/autacoid involved in diverse physiological and pathological processes, is known to interact with various cellular targets such as DNA, thiols, and iron–sulfur proteins, as well as hemoproteins that are either imidazole- or thiolate-coordinated such as the P450s and $^{\bullet}$NO synthases (NOSs) [48–51]. The P450 thiolate-ligation apparently stabilizes its ferric heme state, greatly favoring $^{\bullet}$NO-binding [52]. $^{\bullet}$NO interacts with both the ferric and ferrous forms of P450 enzymes with relatively high affinity, leading to their inhibition [52–59]. By far, the fastest interactions are apparently with P450s exhibiting the highest resting content of high-spin species [52]. This inhibition is short-lived, and initially entails reversible coordination of the NO–nitrogen to the ferric P450 heme iron, but with time, the enzyme is irreversibly inactivated, most likely due to *S*-nitrosylation of P450 cysteine residues [52–59]. These latter adducts are rather long-lived and do not readily dissociate. However, inclusion of dithiothreitol (DTT) earlier on in the incubation can reverse most of this "irreversible" P450 inhibition, whereas inclusion of a thiol source (albumin, but not of a P450 substrate) can partly protect from this inhibition [54, 57]. This biphasic NO-P450 interaction can be monitored via ultraviolet–visible (UV–Vis) and/or EPR spectroscopy. In its initial phase, the ferric-nitrosyl complex with a six-coordinated EPR signature ($g=2.26$) is readily reduced enzymatically or chemically and decreases with the concomitant rise of the five-coordinated ferrous-nitrosyl species ($g=2.00$), stemming from the lability of the Fe-S bond, and with features similar to that of P420 [53, 54, 57]. In the second phase, the proximal Cys ligand released from the prosthetic heme iron, is then *S*-nitrosylated by another $^{\bullet}$NO molecule, resulting in the observed prolonged irreversible P450 inactivation [52, 57, 58]. Very similar $^{\bullet}$NO-P450 heme interactions have been verified by UV–Vis/stopped-flow and Resonance-Raman spectroscopies with the P450s from *Mycobacte-*

rium tuberculosis CYP130 and CYP51 [52]. It is noteworthy, however, that in the presence of O_2^-, •NO can be easily converted at diffusion-limited rates to the potent oxidant and nitrating agent peroxynitrite (PON) that functionally inactivates P450s such as CYP3A4, CYP2E1, CYP2B6, and CYP2B1 via nitration of Tyr residues [50, 60–66] and consequent disruption of their CPR-mediated reduction [2]. In the case of the P450-like endothelial NOS (eNOS), such PON-mediated irreversible inactivation of the enzyme is associated with destruction and loss of the prosthetic heme [59, 63–65].

Various biosynthetic P450 enzymes responsible for the metabolism of endogenous substrates are also targets of inhibition by NO at concentrations physiologically encountered, often with significant pathophysiological consequences [66–74]. Some of these NO-targeted P450 pathways include prostacyclin synthase [66], the renal CYP4A ω-hydroxylase that converts arachidonic acid to vasoactive hydroxyeicosatetraenoic acids (HETEs) [67–69], CYP11A1 (P450scc)-dependent steroidogenic conversion of cholesterol to pregnenolone in Leydig cells [70, 71], 25-hydroxycholesterol and progesterone-stimulated CYP11B1-dependent aldosterone synthesis in bovine adrenal zona glomerulata cells [72, 73], and CYP19A1 (aromatase) function in ovarian steroidogenesis, with consequent impairment of estradiol secretion from human ovarian granulosa cells into the circulation [74]. Thus, by targeting specific P450 biosynthetic pathways, NO can serve as an autocrine regulator.

Ferric P450 species have long been known to also bind sulfur ligands such as thiols (mercaptoethanol, 1-propanethiol, *p*-chlorothiophenol) and sulfides (octyl methyl, pentamethylene, butylmethyl, dibutyl, and methyl phenyl) at its sixth heme-iron coordination site, yielding a unique UV–Vis "hyperporphyrin split Soret" spectral signature, wherein the Soret band exhibits two peaks with maxima around 370–380 and 455–470 nm, respectively [75–78]. Parallel EPR analyses indicated shifts in the existing *g*-values of the P450 complexes, thereby verifying ligand perturbations of the ferric or ferrous heme-iron field on sharing the lone electron pair of the sulfur ligand to form a coordinate bond [78]. Sulfur ligands with bulky hydrophobic side chains can additionally interact at the lipophilic P450 active site, thereby substantially enhancing their P450 binding affinities [78]. In their oxidized ferric states, the sulfide–P450 complexes exhibit red-shifted Soret bands in the 420–470 nm region. Reduction of the heme iron in these P450 complexes with sodium dithionite, reverts the spectra to the characteristic Soret (449 nm maximum absorption with α- and β-bands observed with ligands such as carbon monoxide (CO)) [78]. Furthermore, depending on their relative affinities, such ligands can compete quite effectively with normal substrates and inhibitors such as metyrapone [78]. Hemin-coordinated complexes with mercaptides, phosphines, and thioethers have been examined both by UV–Vis and EPR spectroscopy as models for ferric P450, and confirm these features [76, 79]. A remarkable difference between these chemical model complexes and corresponding P450 complexes is that the former, being thermolabile, survive only at temperatures below −55 °C and thus are much more transient, whereas the latter are relatively stable at room temperature [76]. The reason for this thermolability is apparently the avid proclivity of the low-spin ferric-heme-mercaptide complexes to be reduced at temperatures>−40 °C. It has also been suggested that in solution the mercaptide radicals can easily dimerize to form the disulfide and thus dissociate from the complexes [76].

Similar thiol-binding to ferric CYP3A4 complexes accounts for its functionally relevant interactions with glutathione (GSH) [80], an important intracellular γ-glutamylcysteine-glycine tripeptide that serves as the cofactor for various detoxifying enzymes (peroxidases, GSH-transferases), as well as a nucleophilic antioxidant that traps and thus detoxifies reactive O_2 species (ROS), including free radicals and peroxides, and reactive electrophilic metabolites. Because of this very property, and the assumption that GSH was not only far too large, but also too hydrophilic a molecule to enter the lipophilic P450 active sites, it has been often used in the past as a diagnos-

tic probe to trap reactive metabolites that escape the P450 active site, and thus as an indicator of chemical reactivity external to the P450 active site. However, it appears that some quite large and promiscuous P450 active sites such as that of CYP3A4 (and possibly that of CYP2C8), can accommodate GSH, as determined by the telltale split Soret UV–Vis difference spectrum characteristic of thiol interactions with the sixth ligand of the P450 heme iron [80; K. K. Korsmeyer & M. A. Correia, unpublished observations, 1995]. Furthermore, this GSH-CYP3A4 binding exhibits positive homotropic cooperativity (Hill equation exhibiting an S_{50} of 8.6 mM and a Hill coefficient of 2.2), thereby revealing an additional allosteric effector site for GSH-binding within the CYP3A4 active site [80]. At physiologically relevant GSH concentrations, such GSH-CYP3A4 binding disrupts the substrate homotropic cooperativity assayed via the CYP3A4-dependent O-debenzylation of 7-benzyloxy-4-(trifluoromethyl)-coumarin (7-BFC) and 7-benzyloxyquinoline, as well as that monitored through spectrally detectable substrate binding [80]. However, not all substrate-effector interactions were similarly affected. For instance, GSH increased CYP3A4 binding of 1-pyrenebutanol (1-PB) monitored as its high-spin spectral (type I) complex, but had little effect on the CYP3A4 binding of either α-naphthoflavone or testosterone [80].Given that GSH is routinely included in CYP3A4 reconstitution assays at relatively high concentrations [81], it is to be underscored, that the CYP3A4-heme-iron–GSH interactions detected at 1–10 mM concentrations, while decreasing 1-PB and 7-BFC homotropic cooperativity, failed to competitively inhibit these substrates, and if at all increased their binding affinity (1-PB) and/or their activity (7-BFC) [80]. Such failure of GSH (unlike that of the organic lipophilic thiols and sulfide agents discussed above) to effectively compete out other substrates in functionally reconstituted CYP3A4 systems, may be due to its relatively lower lipophilicity and consequently lower affinity for the lipophilic CYP3A4 active site, coupled with the expected dissociation that ensues from the P450 heme iron upon CPR-mediated reduction and subsequent competition with O_2 binding.

5.2.2 Coordination to P450 Ferrous Heme

The binding of molecular O_2 to the ferrous P450 heme iron is the *sine qua non* critical step in the P450 catalytic cycle. Thus, ligands that can efficiently compete out the O_2 can very effectively block the P450 catalytic cycle and are highly competent inhibitors. Fortunately, among the very first such ligands to be tested during the pioneering days of P450 discovery was CO [82], a neutral ligand already known to bind the heme moieties of hemoglobin and myoglobin with very high affinity, and thus to effectively block their O_2 transport. As in the case of those hemoproteins, CO exclusively binds to the ferrous (reduced) form of P450 through coordination to the heme iron, giving rise to a spectrally detectable ferrous P450–CO complex with an absorption maxima at approximately 450 nm [82], the spectroscopic signature of all cytochrome P450 enzymes (P450, pigment absorbing maximally at 450 nm in the reduced-CO-bound state) [82]. More recently, when it became amply clear that far from being a single entity, multiple P450 families and subfamilies exist, the suffix CYP (for **cy**tochrome **P**450; CYP450 is factually incorrect!) was coined for each of these numbered P450 isoforms [83]. CO binding involves the donation of electrons from the carbon to the iron through a σ-bond as well as back-donation of electrons from the occupied ferrous iron *d*-orbitals to the empty antibonding π-orbitals of the ligand [84]. Early studies with model ferroporphyrins indicated that only those with a thiolate ligand *trans* to the CO yielded the 450-nm absorption, thereby providing key evidence for the presence of a thiolate fifth ligand in P450 [85]. The 450 nm absorption maximum of the ferrous P450–CO complex is proposed by some to reflect the red-shifted hyperporphyrin split Soret peak of the P450 heme iron–CO complex [76].

CO inhibition is a diagnostic test of P450-catalyzed processes, although the sensitivities of different P450 isoforms to CO differ [86] and a few P450-catalyzed reactions are resistant to its inhibition [87–89]. Moreover, the CO sensitivity to inhibition of P450 enzymes such as aromatase (CYP19) [90, 91] and $P450_{scc}$ (CYP11A1) [92] with multistep catalytic cascades, is drastically reduced as they traverse the conformational and ligand states inherent in each of those catalytic processes. The susceptibility of different families to CO inhibition also varies, appearing to decrease in the order CYP2D>CYP2C>CYP3A among the major drug-metabolizing subfamilies of human liver P450 isoforms [86].

5.2.3 Heme Coordination and Lipophilic Binding

Some of the most powerful reversible P450 inhibitors are agents that can simultaneously bind to the lipophilic regions of the active site as well as coordinate tightly to its prosthetic heme-iron atom (Fig. 5.2a). Such dual tethering of the P450 active site confers much greater inhibitory capacity than that observed with agents that exploit only one of these binding modalities. Thus, the potency and effectiveness of such P450 inhibitors is dictated not only by their hydrophobic character but also by the strength of the bond between their heteroatomic lone pair and the heme iron. Accordingly, organic alcohols, ethers, ketones, lactones, and other structures in which an oxygen atom of the ligand coordinates to the heme iron (Fig. 5.1d), or that stabilize the coordination of the distal water ligand, exhibit a Soret maximum at ≈415 nm [38–40] indicative of poor binding and thus are generally weak P450 inhibitors [38–40, 92–97]. By contrast, agents that incorporate both lipophilic moieties that interact strongly with the P450 protein as well as nitrogen-containing aliphatic or aromatic functions that bind the heme iron tightly (Fig. 5.2), displaying a typical “type II ” difference spectrum with a Soret maximum at 430 nm [38–40, 98, 99], are often

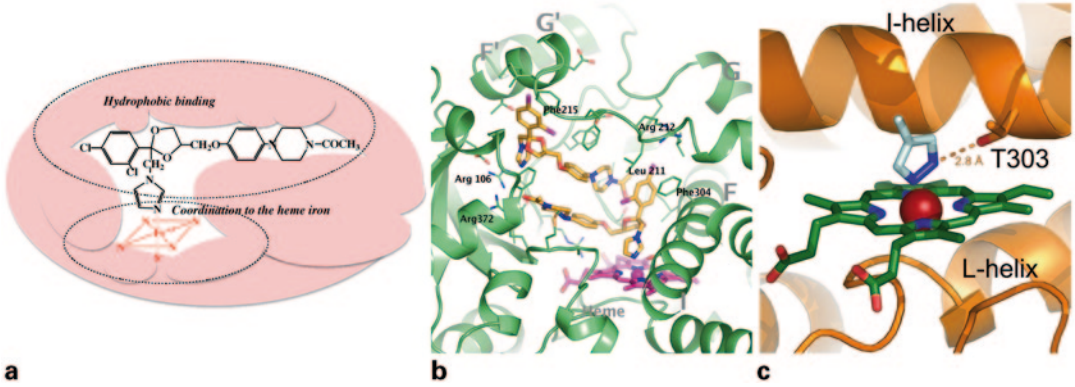


Fig. 5.2 Dual tethering of nitrogenous P450 inhibitors. **a** Ketoconazole (KTZ) bound to the active site of a P450 with a small active site cavity that allows hydrophobic interactions with cavity roof residues as well as coordination to the P450 heme iron, resulting in a potent and highly effective inhibition of the enzyme function; **b** Structural depiction of the much larger CYP3A4 active site with two molecules of KTZ stacked together in an antiparallel orientation, kindly provided by Dr. T. Sjogren [106]. The imidazole nitrogen of the first molecule coordinates with the heme iron (magenta), while its terminal keto group lies in a hydrophilic pocket lined by the Glu374, Arg106, and Arg372 side chains [106]. This interaction is further strengthened through π-stacking hydrophobic interactions with Phe304. The keto group of the second KTZ molecule is H-bonded to the Ser119-side chain, while its chlorobenzyl and imidazole moieties extend towards the protein surface. This snug fit within the capacious CYP3A4 active site makes KTZ a potent FDA-acceptable [36], in vitro CYP3A4 diagnostic probe; **c**Structural depiction of the CYP2E1 active site with a molecule of 4-methylpyrazole (4-MP) coordinated to its heme iron via its pyrazole nitrogen, kindly provided by Dr. E. Scott. 4-MP is shown in blue, heme in green and its iron atom in red. Note the exquisitely snug fit of 4-MP within the relatively smaller CYP2E1 active site cavity lined with its I-helix residues Ala299 and Thr303 [109]

highly effective reversible inhibitors due to these remarkably synergistic features [15–25]. Thus, phenylimidazole, which inhibits P450 much more powerfully than either benzene or imidazole, its individual constituents, provides the simplest example of such synergy [100]. For these reasons, pyridine, imidazole, and triazole moieties have been widely exploited as nitrogenous heterocyclic scaffolds in the therapeutic development of novel P450 inhibitors (Table 5.1) [15–25]. Among the very first of these is metyrapone, an inhibitor of 11β-hydroxylase (CYP11B1), the enzyme that catalyzes the final step in cortisol biosynthesis [101].This feature led to its use as a probe in the diagnosis and treatment of hypercortisolism (Cushing's syndrome) and other hormonal disorders [102].

The inhibitory potency of most reversible P450 inhibitors such as metyrapone and other nitrogenous heterocycles is determined by key structural features such as: (a) the intrinsic affinity of their nitrogen electron pair for the heme iron, (b) the degree to which this intrinsic affinity for the iron is modulated by steric interactions with substituents on the inhibitor [100, 103], (c) the lipophilicity of the nonligating portion of the inhibitor [47, 104], and, obviously, (d) the congruence between the geometry of the inhibitor and the volume of the active site cavity.These structural considerations guided the development of ketoconazole, introduced in 1978 as a "potent, broad-spectrum antifungal agent" (Fig. 5.2a) [105]. However, the recognition that ketoconazole inhibited not just yeast P450 14α-demethylase (CYP51), but also the bifunctional 17-α-hydroxylase/17,20-lyase, CYP17, a key enzyme in androgen-synthesis in the host, led to its therapeutic exploitation in prostate cancer chemotherapy [106]. However, as we now know, ketoconazole also potently inhibits CYP3A4, the major human liver drug metabolizing enzyme, and is in fact the preferred in vitro CYP3A4 diagnostic probe (K_I 0.0037–0.18 μM) recommended by the Food and Drug administration (FDA) [36]. X-ray crystal structural analyses reveal that up to two molecules of ketoconazole can occupy the capacious lipophilic CYP3A4 active site in an antiparallel fashion, with the azole -nitrogen of one of the molecules coordinating the CYP3A4 heme iron tightly [107] (Fig. 5.2b). Not surprisingly then, this CYP3A4 interaction particularly at the higher doses required for cancer chemotherapy, has led to numerous serious DDIs. Indeed, this issue coupled with ketoconazole's potential for severe liver injury and adrenal gland perturbations led the FDA to issue a warning against its therapeutic use in 2013 [108]. Nevertheless, ketoconazole embodies many of the ideal structural features of an effective reversible P450 inhibitor listed above (Fig. 5.2a), and thus has served as an instructive template in the development and structural refinement of other nitrogenous heterocyclic inhibitors that are more selectively tailored to target each specific P450 isoform [20–25]. The quest to further improve on the selectivity and pharmacokinetic properties of ketoconazole has fueled the design and use in antifungal therapy of more potent, selective, and longer lasting CYP51 sterol 14α-demethylase inhibitors such as fluconazole, itraconazole, and terconazole (Table 5.1) [16, 21, 24].

Some of the desirable structural features in an effective reversible P450 inhibitor, such as the individual geometries of the inhibitor and the P450 active site that contribute towards its inhibitory potency, also account for its relative inhibitory selectivity for individual P450 isoforms. They reveal why small molecular weight azole inhibitors such as 4-methylpyrazole (4-MP; Fig. 5.2c) and indazole (INZ) [109] are relatively potent, albeit reversible inhibitors of CYP2E1, a P450 with a relatively small active site cavity (≈190 Å^3) [109, 110], but not of the much more voluminous CYP3A4 (950–1650 Å^3) [107, 111, 112]. Both 4-MP and INZ have been shown to coordinate the CYP2E1-heme iron through one azole nitrogen and to hydrogen bond through the adjacent nitrogen with the side-chain hydroxyl of the conserved Thr_{303} (the only polar group in an otherwise globular and highly nonpolar active site cavity), with the 4-MP methyl (Fig. 5.2c) and the INZ aryl resting snuggly against the lipophilic active site roof [109].

The exploitation of azoles and other nitrogenous heterocycles as scaffolds is by no means restricted to the intentional development of selective P450 inhibitors as therapeutic agents (Table 5.1).

Their widespread incorporation into a variety of other therapeutic agents is also the inadvertent cause of many unwarranted and undesirable DDIs [20, 33, 113–117]. Accordingly, cimetidine, once a popular over the counter H2-antagonist used in gastric ulcer therapy, was found responsible for many DDIs stemming from its imidazole-mediated inhibition of the metabolism of co-administered drugs [118]. This inadvertent side effect prompted the search for, and successful development of, non-imidazole containing H2-antagonists such as ranitidine that are devoid of this undesirable side effect [118, 119], as well as proton pump inhibitors such as omeprazole, with considerably lower incidence of similar DDIs [120].

More recently, inhibitors of specific kinases in the cellular signaling cascades that contain nitrogenous heterocyclic moieties (i.e., quinazoline, quinolone, aminopyridine, aminothiazole, indizole, etc.) have been developed and clinically tested as chemotherapeutic adjuvants in the treatment of various cancerous malignancies [33, 113–117]. Many of these have been shown to interact with P450s such as CYP3A4 and CYP2C8 through type II and "type II-like" spectral interactions, as well as time-dependent P450 inhibition [33, 113–117]. Initial clinical trials of pazopanib (Votrient, an oral antiangiogenic drug, known to inhibit tyrosine kinases of vascular endothelial growth factor (VEGF)-receptor, platelet-derived growth factor receptor, and c-KIT) indicated that the hepatic CYP2C8- and CYP3A4-dependent clearance of chemotherapeutic drugs such as paclitaxel was significantly inhibited [114]. This suggests that in addition to their intrinsic pharmacological utility, cancer chemotherapeutic adjuvants such as pazopanib may provide additional benefits by permitting dosage reduction of coadministered chemotherapeutic drugs with a narrow therapeutic index such as paclitaxel.

It must be underscored that until the advent of more potent and specific mechanism-based inactivators for targeting various P450s of pathologic relevance (i.e., CYP19 /aromatase in breast cancer; CYP17/17,20-lyase in prostate cancer), the structural exploitation of nitrogenous scaffolds (metyrapone, aminoglutethimide, imidazoles, and triazoles) with the objective of improving their potency as well as P450 isoform selectivity was an enterprise of considerable therapeutic interest. This exercise led to the development and clinical testing of pyridylaminoglutethimide, Fadrozole [CGS 16949A {4-(5,6,7,8-tetrahydroimidazo-[1,5-α]pyridin-5-yl) benzonitrile}], Letrozole [CGS 20267, [4,4′-(1H-1,2,4-triazol-1-yl-methylene)-bis-benzonitrile)], CGS 18320B bis-(*p*-cyanophenyl)imidazo-1-yl-methane hemisuccinate, and R-76713 [6-(4-chlorophenyl)1H 1,2,4 triazol-1-yl)-methyl]-1-methyl-1H-benzotriazole as nonsteroidal aromatase inhibitors [16, 121–123] (Table 5.1). Indeed, Letrozole once represented a highly promising imidazole as a second line of hormone ablative therapy in patients with hormone-dependent breast cancer [123].

5.2.4 Type II Versus Pseudo Type II Spectral Interactions

It has long been assumed that nitrogenous heterocycles that interact with the P450 heme iron yielding a type II difference spectrum, also confer greater metabolic stability to the complex than type I ligands, and thus may be viewed essentially as P450 inhibitors (Fig. 5.1b). This, as discussed earlier, is because the low-spin character of the P450 heme iron in such type II complexes raises its redox potential, thereby impeding CPR-mediated reduction [41, 42]. However, more recent evidence in the literature indicates that this notion must be revised. Studies of a synthetic chemical library based on a quinoline carboxamide (QCA) structural scaffold with benzene, toluene, anisole, or *N*,*N*-dimethylaniline at the amide position and benzene, pyridine, pyrimidine, or pyrazine at the two-position of the quinoline ring indicated that while some of these substituted QCA analogs yielded type I spectral interactions with CYP3A4, others yielded essentially type II or type II-like spectral interactions with the enzyme [124–126]. Yet, far from being metabolically stable "dead-end" complexes, the latter not only exhibited respectable reduction rates but also in vitro intrinsic metabolic clearances (V/K) that were up to 12-fold higher than

those of the corresponding structural QCA analogs yielding type I spectra. Thus, in spite of exhibiting type II spectral interactions, QCAs were quite efficiently metabolized by hepatic P450s at subsaturating concentrations [124–127].

Instructive insight into this conundrum was provided through scrutiny of individual CYP3A4-heme iron interactions with 17-ethinylestradiol (EE), a well-recognized suicide substrate, and its 1,2,3-triazole (1,2,3-TRZ) derivative (1,2,3-TRZ incorporated at the EE-D-ring via "click" chemistry), through differential UV–Vis spectroscopy, continuous-wave electron paramagnetic resonance (EPR) and hyperfine sublevel correlation spectroscopy (HYSCORE) EPR spectroscopy [128]. Upon EPR analyses, CYP3A4-heme iron–EE complexes were indeed found to be high-spin, consistent with their type I spectral interaction, and this was further verified by HYSCORE EPR analyses that revealed the inherent displacement of water from the prosthetic heme-iron sixth axial ligand. Corresponding analyses of CYP3A4-heme iron–1,2,3-TRZ-EE complexes revealed a type II -like spectral interaction, but surprisingly no alteration of the spin state or water displacement from the basal water-ligated CYP3A4-heme-iron complexes (Fig. 5.1c) [128]. This was in complete contrast to the binding of authentic type II ligands such as imidazole or triazole (Fig. 5.1b) or 1,2,3-TRZ to CYP3A4 [128]. Thus, CYP3A4-heme iron–1,2,3-TRZ-EE complexes were found as water-bridged low-spin complexes that were metabolically competent, as verified by their ability to generate D-ring hydroxylated EE-derivatives [128]. A similar water-bridged complex was also observed in the crystal structure of *M. tuberculosis* CYP121 with fluconazole, an antifungal 1,2,4-TRZ-derivative [129]. Close inspection of the difference spectral data, however, revealed a remarkable feature of the CYP3A4–1,2,3-TRZ-EE complexes relative to corresponding CYP3A4 complexes with either imidazole or 1,2,3-TRZ that exhibit a Soret maximum at 424 or 422 nm, respectively, and thus are red shifted from the absolute CYP3A4 spectrum (416 nm Soret maximum) by 6 and 8 nm, respectively [128]. The Soret red shift of the CYP3A4–1,2,3-TRZ-EE complex on the other hand was of the order of only 2 nm. Furthermore, the diminution of the CYP3A4 high-spin fraction (Δabs_{390} nm) in this complex was 0.34 relative to 1.0 in the CYP3A4–1,2,3-TRZ complex, with an even greater reduction in the peak minus trough intensity of the calculated difference spectrum relative to that of the CYP3A4–1,2,3-TRZ complex [128]. Thus, unlike the authentic type II spectral signatures of CYP3A4-1,2,3-TRZ and imidazole, that of the CYP3A4–1,2,3-TRZ-EE complex was more "type II-like" [128]. Inspection of the spectral interaction data of the metabolically competent QCA analogs indeed reveals that this "pseudo" type II spectral signature with diminished amplitude of spectral intensity and minimal Soret red shift is also their common feature. This was also true of all the other type II-like complexes of the P450 isoforms other than CYP3A4 examined [127, 128]. It is striking that this particular spectral signature resembles essentially that of the "modified type II" or "reverse type I" binding of the P450 heme-iron sixth ligand by organic alcohols and ketones [39, 130, 131]. Importantly, the identification of this "pseudo" type II spectral signature is a valuable diagnostic tool in the preclinical assessment of potential novel drug candidates bearing nitrogenous heterocyclic pendants as either P450 substrates or inhibitors .

5.3 Catalysis-Dependent Inhibition

A significant number of different classes of compounds are known to contain functional groups that have been shown to predispose the molecule to metabolism by particular cytochrome P450 isozymes to form reactive intermediates that can either quasi-irreversibly or irreversibly inactivate the enzyme responsible for their formation. This irreversible inactivation by the reactive species generated catalytically is routinely superimposed on reversible inhibition of the P450 due to competitive binding of the parent compound to the P450 active site. Compounds that inactivate enzymes in this fashion either irreversibly or quasi-irreversibly are considered to be mechanism-based (catalysis-dependent, suicide, or time-dependent) inactivators [132, 133]. Key to this

concept of mechanism-based inactivation (MBI) is the requirement that the inactivation involves formation of a covalent adduct with the protein or the heme prosthetic group without the release of the reactive intermediate from the protein into the medium. As a consequence, this definition rules out affinity labels, transition state analogs, and slow, tight binding inhibitors. As pointed out previously by Correia and Ortiz de Montellano [24], and underscored above, mechanism-based inactivators are much more enzyme specific than reversible inhibitors. The reasons for this are as follows: (a) the initial binding of the mechanism-based inhibitor by the enzyme must satisfy all of the constraints imposed on reversible inhibitors; (b) the mechanism-based inactivator must also be able to function as a substrate since it must undergo catalytic activation to form a reactive species; and (c) the resulting reactive intermediate formed as a consequence of the catalytic reaction must then find an appropriate target within the enzyme active site or in an access or egress channel to the active site and react with it, leading to irreversible modification of the protein or the heme, which then permanently removes that molecule from the pool of active enzymes. The four general classes of mechanism-based inactivators of P450s include: (a) compounds that bind quasi-irreversibly to the iron atom of the prosthetic heme; (b) agents that covalently modify the porphyrin framework of the heme; (c) compounds that lead to the destruction of the prosthetic heme group with consequent irreversible modification of the P450 active site by the ensuing heme fragments; and (d) compounds that form covalent adducts to amino acid residues in the apoprotein. It should be noted that mechanism-based inactivators may concurrently inactivate by more than one mechanism, and the mechanism that predominates for any given inactivator may be determined by a number of factors, including the identity of the enzyme responsible for the formation of the reactive intermediate and the presence of other proteins such as b_5 that may affect the catalytic trajectory and the three-dimensional structure/conformation of the active enzyme. So far, the factors that determine how a specific mechanism-based inactivator modifies a particular P450 enzyme are not well understood.

5.3.1 Quasi-irreversible Coordination to the Prosthetic Heme

Certain P450 substrates containing either a methylenedioxyphenyl (MDP) functionality that is biotransformed to an electrophilic carbene moiety, or organic amines that are oxidized in situ to nitroso products, coordinate so tightly to the ferrous P450 heme-iron atom so as to become virtually irreversible, except under very special experimental conditions [134–139]. Such substrate-derived "metabolic-intermediate (MI) complexes" requiring initial P450 catalytic turnover for their generation are both functionally incompetent and long-lived, effectively aborting further P450 catalytic recycling, and resulting in potent, highly efficient and long lasting P450 inhibition, and consequent clinical DDIs. However, such an effective "freezing" of the P450 heme iron also aborts its oxidative turnover, the root of the normal "wear and tear" of the P450 protein, and thus a key determinant of its cellular disposal and physiological half-life. As a result, P450s engaged in these long-lived MI complex es accumulate over time and are "induced via stabilization" [140–142]. However, because neither the P450 heme nor the protein moiety is irreversibly damaged in these MI complexes, and they are actually quasi-, rather than fully irreversible, it is plausible that under certain physiological circumstances such elevated levels of hepatic P450s, on release from their MI complex-bondage, could become functionally active and thus contribute to clinically relevant DDIs.

5.3.1.1 Methylenedioxyphenyl Compounds

Aryl and alkyl MDP compounds, many present naturally in oils, spices, and medicinal herb supplements [143–146], and/or used either as therapeutic drugs or insecticide synergists [134–139], are oxidatively transformed by P450 enzymes to reactive intermediates that coordinate

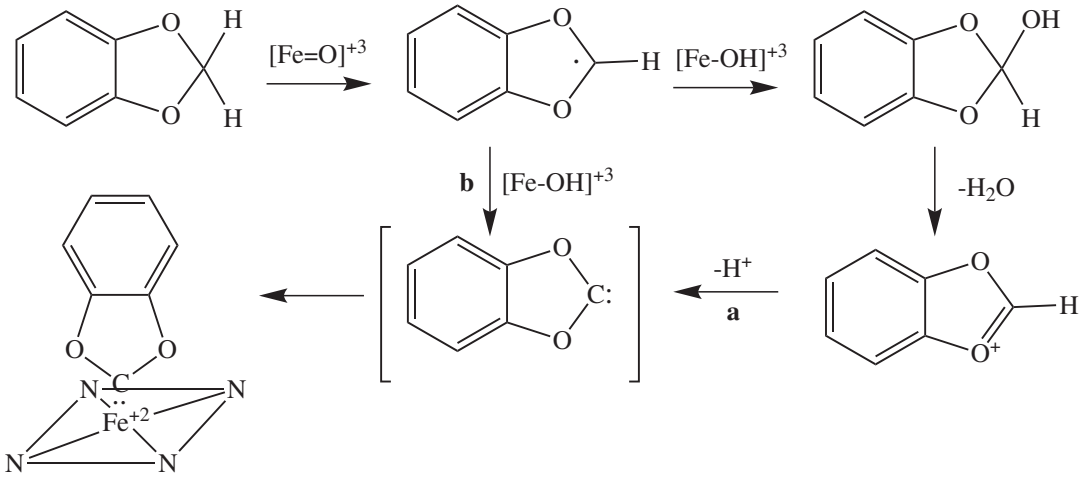


Fig. 5.3 Mechanism of quasi-irreversible mechanism-based inactivation via P450-catalyzed MI-complex generation from the MDP compounds. P450-mediated oxidation of the MDP moiety results in the formation of a carbene species that coordinates tightly to the P450 heme iron. See the text for mechanistic details. *MI*, metabolic intermediate; *MDP*, methylenedioxyphenyl

tightly to their heme iron atom [136] yielding MI complexes (Fig. 5.3).Such MI complex formation not only is time-, e NADPH, O_2-, and concentration-dependent but can also be initiated with cumene hydroperoxide, instead of NADPH and O_2, thereby verifying the vital role of P450 catalytic turnover in this process [134, 147, 148]. The resulting ferrous complex typically exhibits a difference absorption spectrum with maxima at 427 and 455 nm, whereas the corresponding ferric complex exhibits a single absorption maximum at 437 nm [134, 137]. The peaks at 427 and 455 nm apparently are due to structurally distinct ferrous complexes, although their interrelationship remains obscure [136]. The ferrous complex is relatively stable and can be isolated intact from animals treated with isosafrole, whereas the ferric complex is less stable and can be easily disrupted upon incubation with lipophilic compounds, thereby regenerating the catalytically active enzyme [149, 150]. By contrast, the ferrous complex is resistant to incubation with lipophilic compounds, but can be disrupted by irradiation at 400–500 nm [151, 152]. As in the case of reversible inhibitors such as ketoconazole (Sect. 5.2.3), concurrent binding interactions of the ligand with the lipophilic active site stabilize the ferrous P450 complex [153]. Accordingly, structure activity studies of 4-alkoxy-1,2-methylenedioxybenzene reveal that the size and lipophilicity of the alkoxy group is an important determinant of the corresponding MI complex stability: Alkyl chains of 1–3 carbons yield unstable MI complexes whereas those with longer alkyl groups are relatively more stable [153, 154]. Transition from the ferrous to the ferric state weakens the complex, indicating the preference of the reactive MDP-derived species for strong coordination to the ferrous iron, much like CO.

The above findings, together with the characterization of model synthetic carbene complexes [155, 156], provide a compelling argument for the catalysis-dependent generation of a carbene–iron complex (Fig. 5.3). The striking structural resemblance of a carbene to CO readily accounts for the unusual 455-nm absorption maximum of the MDP-derived MI complex, and its designation as a bona fide carbene complex. The nature of the MDP-derived complex with a spectral absorption maximum at 427 nm is presently less clear, but may reflect a MDP-derived carbene complex devoid of its thiolate ligation as in P420, or another as yet unidentified *trans* ligand [157]. The observed incorporation of O_2 from the medium into the CO metabolite derived from the MDP bridge carbon (see below), and the observa-

tion that CO formation is enhanced by electron-withdrawing substituents, further rationalize the intrinsic carbene nature of the MDP–MI complex [158]. The structural intermediacy of the carbene in the MDP–MI complex is also further strengthened by the observation that addition of water to the iron-coordinated carbene produces an iron-coordinated anion that, as expected, decomposes into the observed catechol and CO metabolites. The observed incorporation of an atom of molecular O_2 into a fraction of the MDP-derived CO, on the other hand, is less transparent and awaits a mechanistic explanation [158].

The currently solid link between the MDP moiety and P450 inhibition, the requirement for P450-mediated metabolic activation of the MDP inhibitor, and the fact that the MDP bridge carbon is indeed the target of this oxidation, leave little doubt of its critical role in this inhibition. Although free radical [159], carbocation [160] and carbanion [151] intermediates have been implicated, it is evident that the formation of the carbene from the bridge-hydroxylated MDP metabolite, or from its radical precursor, is most consistent with all the available experimental evidence (Fig. 5.3). The key role of the MDP group is further strengthened by the fact that substituents other than an alkoxy group on the MDP moiety suppress complex formation [135, 136, 161]. The accessory role of an alkoxy substituent is mechanistically sound, given that its O-dealkylation would provide an independent route to the bridge-hydroxylated precursor of the carbene [152]. Furthermore, additional evidence for a protagonistic role of MDP bridge hydroxylation in P450 inhibition may be derived from the findings that aryldioxymethylenes are oxidized to catechols, carbon monoxide, carbon dioxide, and formic acid [135, 158, 162–164], and from the observation that deuterium substitution on the MDP-bridge carbon decreases the rate of CO formation ($k_H/k_D = 1.7–2.0$). A similar isotope effect encountered in the in vivo insecticide synergizing activity of these compounds firmly confirms the mechanistic association between the formation of CO and the MI complex, with consequent P450 inhibition [165].

Three mechanistic pathways are plausible for oxidation of the MDP–dioxymethylene bridge to the iron-coordinated carbene: In the first, hydroxylation of the dioxymethylene bridge followed by elimination of a water molecule results in an acidic oxonium ion that upon deprotonation gives the carbene (Fig. 5.3, path a).In the second, formation of the oxonium species could precede generation of the bridge-hydroxylated metabolite, if the ferryl species were to oxidize the radical formed in the hydroxylation reaction before the oxygen rebound occurs (Fig. 5.3, path b). In the third, the same radical intermediate could bind to the iron of the $[Fe\text{-}OH]^{3+}$catalytic intermediate [155]. Subsequent deprotonation and intramolecular transfer of the oxygen from the iron to the carbon would yield the bridge-hydroxylated metabolite that could then decompose to the carbene complex as in *path a*. Regardless of the precise chemical mechanism of MI complex formation, the elucidation of potent and long-lasting P450 inhibition via MDP-mediated MBI has provided mechanistic rationales for the beneficial exploitation of piperonyl butoxide and other similar MDPs as insecticide synergists [134, 135, 162, 163], as well as for the potential of adverse clinical DDIs upon therapeutic MDP-containing drug coingestion.

Several clinically prescribed drugs and once prospective drug candidates contain the MDP scaffold (Fig. 5.4). One example is paroxetine (Fig. 5.4), a selective serotonin reuptake inhibitor (SSRI) [166–173]. In vitro studies with purified CYP2D6 indeed reveal the formation of MI complex es with the characteristic spectroscopic signature at 456 nm [172]. The intermediacy of a carbene is further supported by the fact that paroxetine is metabolized by CYP2D6 via demethylenation of the MDP group to a catechol and formic acid [169, 173]. The K_I and k_{inact} values of 6.6 ± 2.7 μM and 0.25 ± 0.09 min^{-1}, respectively, calculated for the paroxetine-mediated inhibition of human liver microsomal CYP2D6-dependent dextromethorphan O-demethylation [172], are fully consistent with clinical reports of its potent CYP2D6 inhibition [166–173]. Another noteworthy example of an MDP-bearing drug is noscap-

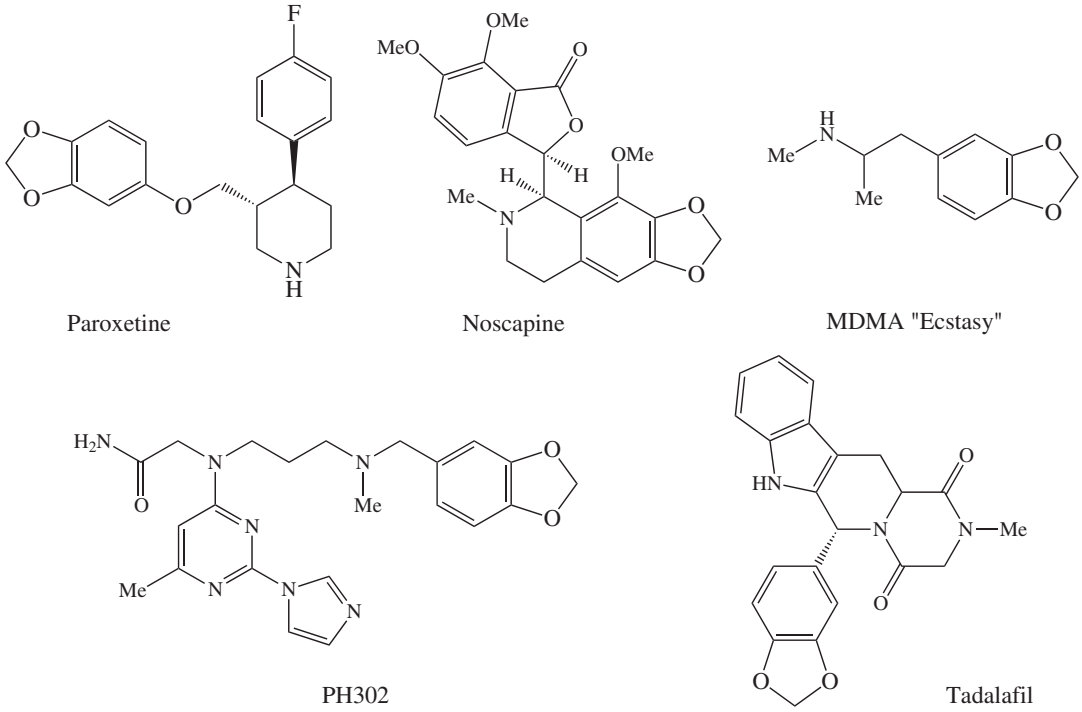


Fig.5.4 Therapeutic, designer, and would-be drugs as examples of MDP compounds documented to be quasi-irreversible inactivators of certain P450 isoforms via MI complexation. *MDP* methylenedioxyphenyl, *MI* metabolic intermediate

ine (Fig. 5.4), a nonaddictive, phthalideisoquinoline alkaloid derived from the opium poppy latex, and widely recognized as a safe and promising cough suppressant as well as a potential cancer chemotherapeutic agent when administered at much higher doses [174–177]. At antitussive doses, significant clinical DDIs of noscapine were reported with the anticoagulant warfarin, a drug with a relatively low therapeutic index [177–179]. Indeed, in vitro studies with human liver microsomes (HLMs) and purified recombinant wild-type CYP2C9 (CYP2C9.1 variant) revealed time-dependent inactivation of CYP2C9-mediated *S*-warfarin 7-hydroxylation with concomitant 458-nm MI-complex formation [174]. Intriguingly, CYP2C9.2 and CYP2C9.3 allelic variants were even more efficiently inactivated by noscapine, with a >twofold increase in k_{inact}/K_I, thereby revealing the additional potential for further aggravated CYP2C9-genotype-dependent DDIs, particularly upon ingestion of the much higher noscapine doses required for cancer chemotherapy [174]. Yet another example of a therapeutic MDP drug is the phosphodiesterase-5 inhibitor, tadalafil (Cialis; Fig. 5.4), currently used for the treatment of erectile dysfunction [180]. Although in vitro assays by the manufacturer revealed that tadalafil indeed caused time- and concentration-dependent MBI of CYP3A4-dependent midazolam 1′-hydroxylation with a k_{inact} of 0.21 ± 0.004 min^{-1} and a K_I of 12 ± 0.4 μM, the drug was thought to be of sufficiently low potency to be of any signficant concern in clinical DDIs [180]. Furthermore, studies in healthy volunteers by the same team indicated no significant DDIs between midazolam and lovastatin, two CYP3A4 substrates, after ingestion of a single oral dose of tadalafil [180]. Although tadalafil ingested at the recommended dosage was thus exonerated from any potentially meaningful DDIs, the concern remains that given its relatively long half-life of 17.5 h, it may not be quite as innocu-

ous if ingested in an accidental overdose and/or in combination with other CYP3A4 inhibitory drugs such as macrolide antibiotic s, azole antifungals, or HIV protease inhibitors [181].

Yet another noteworthy example is the widely abused MDP-containing amphetamine-based designer drug MDMA (*N*-methyl-3,4-methylenedioxyamphetamine, "Ecstasy" or "Adam"; Fig. 5.4). Due to the initially limited experimental focus on just CYP2B enzymes, its potential for MBI was long overlooked and it was thought, in fact, to not engage in any P450–MI complex ation [182]. It was only more recently that the principal role of CYP2D6 in its metabolism and consequent MBI was identified [183–190]. Indeed, MDMA inactivated recombinant yeast microsomal CYP2D6-dependent dextromethorphan O-demethylation in a time- and concentration-dependent process with a k_{inact} and K_I of 0.29 ± 0.03 min^{-1} and 12.9 ± 3.6 μM, respectively. Three HLM preparations, genotyped as extensive CYP2D6 metabolizers, similarly yielded k_{inact} values ranging from 0.12 ± 0.05 to 0.26 ± 0.02 min^{-1}, and corresponding K_I values ranging from 14.4 ± 2.5 to 45.3 ± 32.1 μM [184]. Difference spectral analyses with recombinant yeast microsomal CYP2D6 also yielded the telltale spectral signature of a 456-nm MI complex. In vivo, MDMA-elicited MBI apparently occurs promptly within 2 h of a recreational dose, and recovery to basal levels requires at the least 10 days [188, 189]. While DDIs with the MDP-paroxetine have been documented, life-threatening DDIs also occur with other CYP2D6 inhibitors such as the HIV-protease inhibitor ritonavir (RTV) and monoamine oxidase (MAO) inhibitors [190, 191]. Much less is known about any similar MBI potential of the other illicit designer drugs such as MDE (*N*-ethyl-3, 4-methylenedioxyamphetamine, or "Eve"), MDA (3, 4-methylenedioxyamphetamine) and the pure cocaine-like MDP-psychostimulant methylenedioxypyrovalerone (MDPV).

The highly active and widely used cancer chemotherapeutic epipodophyllotoxins etoposide and teniposide are also MDP-containing glycosides metabolized primarily by CYP3A4, and to a lesser extent by CYP2E1 and CYP1A2 [192, 193], but their potential for MI complexation was not addressed and similarly remains to be defined. Dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-dicarboxylate (DDB), an intermediate in the natural synthesis of Schizandrin C in *Fructus Schizandrae chinensis,* is a hepatoprotective agent against a variety of liver injuries, including alcohol-induced steatosis, that is widely used in Asia [194]. Of all the human liver P450 isoforms evaluated with diagnostic probes, it was found to potently inactivate CYP3A4-dependent testosterone 6β-hydroxylase with an IC_{50} value of 0.38 μM [194]. When incubated in vitro with liver microsomes from pregnenenolone 16α-carbonitrile (PCN)-pretreated rats, it yielded a spectral maximum at ≈458 nm, characteristic of an MI complex [194].

The MDP-pyrimidineimidazole compound PH302 (Fig. 5.4) is a potent and selective inhibitor of the inducible •NO synthase (iNOS), acting via coordination to the iNOS-monomeric heme moiety so as to prevent dimerization of the protein [195]. Upon preclinical absorption, distribution, metabolism, and excretion (ADME) screening, it was eliminated as a drug candidate when it was found to also inhibit CYP3A4 rather potently [196]. Interestingly, PH302 serves as a highly illustrative example of a P450 inhibitor with dual spectrally detectable mechanistic features: It forms a type II complex with recombinant CYP3A4 and competitively inhibits CYP3A4-dependent midazolam and testosterone hydroxylations with a K_I of ≈2 μM [196]. However, at maximal PH302 concentrations, the maximal type II shift is only 72% of that observed with imidazole at saturating concentrations [196]. By virtue of its MDP moiety, it also is a CYP3A4 mechanism-based inactivator, exhibiting unusual biphasic characteristics: An initial fast phase (0–1.5 min) with a k_{inact} of 0.08 $min^{-1}$and K_I of 1.2 μM, and a second phase lasting 1.5–10 min, with a k_{inact} of 0.06 min^{-1} and K_I of 23.8 μM. Interestingly, the difference spectrum resulting from these interactions exhibited both type II as well as MI complex spectral features, thereby revealing the simultaneous occurrence of dual P450 binding modes [196]. Its inherent MDP activation to a carbene complex is consistent with

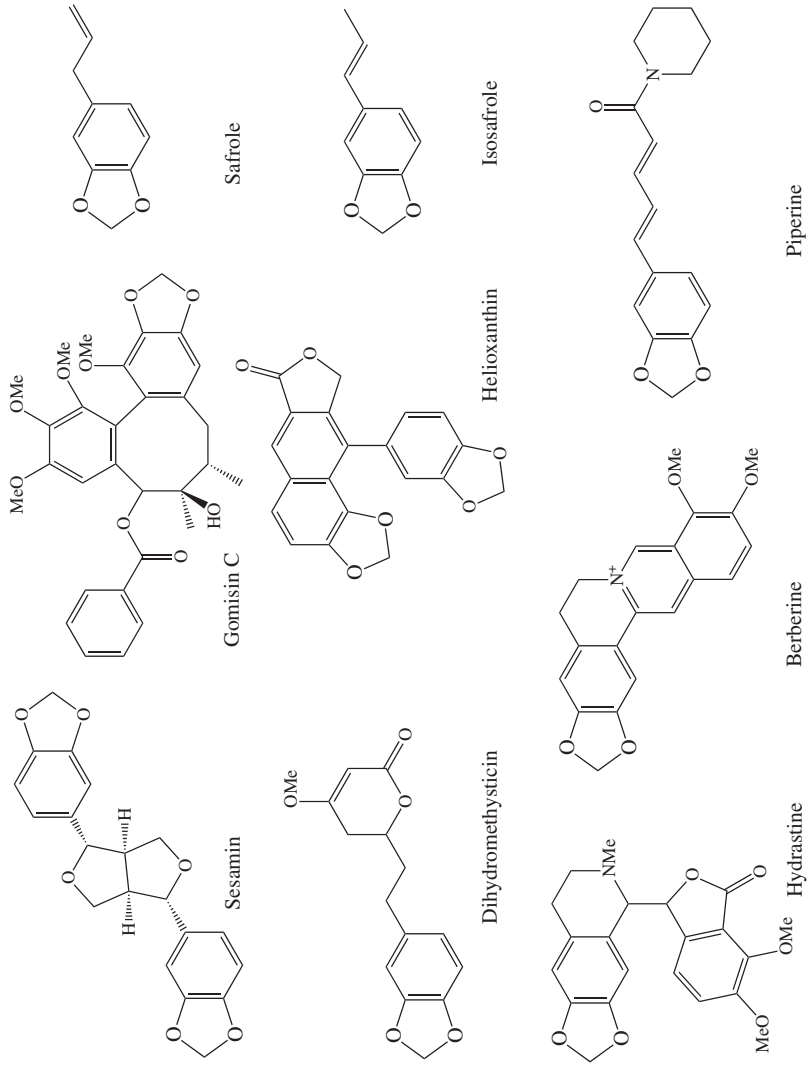


Fig. 5.5 Examples of MDP compounds naturally present in herbal remedies, oils, and spices documented to be quasi-irreversible inactivators of certain P450 isoforms via MI complexation. Note that not only the concentrations of some of these MDP compounds required to inactivate P450s may exceed the levels present naturally, but also may vary widely from batch to batch

the concurrent detection of a catechol metabolite [196].

Reports of adverse herb-drug interactions upon intentional or accidental coingestion of high enough doses of MDPs naturally present in dietary supplements, ritual beverages, and traditional phytotherapeutic medicines (Fig. 5.5) are also clinically abundant [143–146]. Safrole and isosafrole present (Fig. 5.5) in oil of *Sassafras,* once used as a root-beer flavoring agent, but now banned because of its carcinogenic potential, rank among the first discovered MDPs as P450 inhibitors [197–200]. Isosafrole, a precursor in the chemical manufacture of the fragrance heliotropin (piperonal) and the recreational psychostimulant MDMA, is a mechanism-based substrate/inactivator of CYP1A2 that in rats was found to produce a stable, isolable CYP1A2–MI complex and thus to "induce" CYP1A2 via stabilization [141].

Sesame oil also contains several MDPs such as the antioxidant sesamol, and lignans such as the dietary fat-reducing supplement sesamin (Fig. 5.5) and sesamolin [2001]. Although CYP2C9 and CYP1A2 metabolized sesamin to its monocatechol metabolite, only CYP2C9 underwent MBI, most likely via an MI complex with apparent K_I and k_{inact} values for diclofenac-4′-hydroxylation of 22 μM and 0.13 min^{-1}, respectively [201, 202]. Sesamin was also reported to potently inhibit CYP3A -dependent metabolism of α- and γ-tocopherols to their corresponding 3′- and 5′-δ-carboxychroman metabolites in HepG2 cells [203], although it is unclear whether such inhibition involves an MDP-associated MBI. The MDP-lignans [(−)clusin, (−)dihydroclusin, (−)yatein, (−)hinokinin, and (−)dihydrocubebin] isolated from *Piper cubeba* were all found to cause a potent and selective CYP3A4 MBI that was time-, concentration-, and NADPH-dependent [204]. (-)Clusin and (-)dihydroclusin ranked as the most potent of these with K_I values of 0.082and 0.054 μM and k_{inact} values of 0.253 and 0.310 min^{-1}, respectively [204]. All these MDP-compounds yielded the telltale spectrallydetectable, NADPH-dependent 455-nm MI complex [204]. Time-, concentration-, and NADPH-dependent CYP3A4 MBI was also documented with the MDP-lignans (savinin, helioxanthin; Fig. 5.5) and 3-(3″,4″-dimethoxybenzyl)-2-(3′,4′-methylenedioxybenzyl)butyrolactone from *Acantho-panenax chiisanensis* (stems and bark used as a tonic and sedative as well as antirheumatoid arthritis remedies), with K_I values of 2.4, 1.6, and 2.2 μM and k_{inact} values of 0.030, 0.043, and 0.047 min^{-1}, respectively [205].

The kavalactones in the anxiolytic extracts of kava kava *Piper methysticum* include methysticin (M) and dihydromethysticin (DHM; Fig. 5.5) that in vitro exhibit the characteristic 455-nm MDP–MI complex spectral signature with P450s present in HLMs [206]. Functional assays with relatively selective P450 diagnostic probes revealed that M inhibited CYP2C9 by 58%, CYP2D6 by 44%, and CYP3A4 by 27%, whereas DHM was found to inhibit CYP2C19 by 76%, CYP2C9 by 69%, and 3A4 by 54% [206]. Kava coingestion with other drugs, including sedative-hypnotics, barbiturates, and benzodiazepines (alprazolam; [207]), is associated with significant DDIs [206, 208–210], and studies in healthy volunteers also identified CYP2E1 as another target [211]. Similarly, the goldenseal (*Hydrastis canadensis*) MDP alkaloids berberine and hydrastine (Fig. 5.5) present at comparable levels in extracts popularly used as a medicinal immunostimulant against common cold and upper respiratory tract infections, were shown to inhibit CYP2C9-dependent diclofenac-4′-hydroxylation, CYP2D6-dependent bufuralol-1′-hydroxylation, and CYP3A4-dependent testosterone 6β-hydroxylation [212]. Of the two MDP alkaloids, berberine was the most potent against CYP2D6, exhibiting an IC_{50} value of≈45 μM, whereas it was the least inhibitory of CYP3A4 (with an IC_{50} value of≈400 μM). On the other hand, the (+) and (−) hydrastine isomers were only weakly inhibitory towards CYP2D6 (with an IC_{50} value of≈350 μM for each isomer), but inhibited CYP3A4 with K_I values of 25 and 30 μM, respectively [212]. An apparent K_I value of ≈110 μM and a k_{inact} value of 0.23 min^{-1} was determined for the NADPH-dependent CYP3A4 MBI by (−) hydrastine [212]. Both hydrastine isomers formed spectrallydetectable MI complexes with CYP3A4, CYP2D6, and CYP2C9, and the rate of these CYP3A4 and CYP2C9 MI complexes was significantly increased in the presence of b_5 [212]. The clinical relevance of these in vitro findings is underscored by studies in healthy volunteers that showed that oral goldenseal ingestion in the form of hard gelatin capsules (64.8 mg hydrastine, 77.4 mg berberine; 142.2 mg total MDP alkaloid /day) indeed strongly inhibited the metabolism of CYP3A4 and CYP2D6 diagnostic probes in vivo [211].

Other natural MDP compounds of note include the sedative-antitussive gomicin C (isolated from the *Schisandra* fruit extracts; Fig. 5.5) and shown to cause a potent time- and concentration-dependent MBI of CYP3A4 (K_I value of ≈0.399 μM and a k_{inact} value of 0.092 min^{-1}) that is associated with spectrally detectable MI complexes [143, 213]. The K_I value of gomicin C as a competitive inhibitor of CYP3A4 (0.045 μM) is lower than that of ketoconazole (0.070 μM), the commonly

employed CYP3A inhibitory probe, reflecting its relatively tighter binding and greater inhibitory potency [143, 213]. Piperine (Fig. 5.5), an alkaloid present in black pepper (*Piper nigrum*) and traditionally exploited as an antidiarrheal remedy, is a mixed-type competitive inhibitor of CYP3A4 in in vitro studies with both HLMs and recombinant enzyme [145, 214–216]. Piperine and other MDP alkaloids from black pepper were also found to elicit a potent MPI of CYP2D6 in vitro [217, 218]. Indeed, piperine also inhibited the metabolism of CYP2D6-diagnostic probes (propranolol [219] and spartein [220]) in human volunteers in vivo, thereby underscoring its potential for clinically relevant DDIs. Piperonylic acid, another MDP-containing natural product extracted from the bark of the Paracoto tree is also found to selectively and potently inactivate CYP73A1-dependent *trans*-cinnamic acid 4-hydroxylase via MI-complex formation in vitro with a $K_I = 17$ μM, and a $k_{inact} = 0.068$ min^{-1} [221]. Such quasi-irreversible inactivation of the core phenylpropanoid pathway was also shown to occur in vivo in tobacco leaves and cell cultures [221].

Additional MDP compounds have been synthesized and their human isoform selectivity as mechanism-based inactivators evaluated [222]. Their inactivating potential depends on the side-chain structure, with bulky side chains such as 1,4-benzothiazine inactivating some P450 enzymes but not others [222]. P450 heme iron–carbene complexes are also involved in the anaerobic reductive coordination of halocarbons to the heme-iron atom, but this reaction, as discussed previously (24), is linked to destruction of the prosthetic heme.

5.3.1.2 Alkyl/Aryl Amines

A second large class of agents known to form mechanism-based quasi-irreversible P450–MI complex es [15, 24, 25, 138, 139, 223–228] includes alkyl and aromatic amines, such as the monoamine oxidase inhibitor clorgyline [223], the SSRI sertraline [228], and many clinically useful macrolide antibiotics such as troleandomycin (TAO), clarithromycin, and erythromycin (Fig. 5.6; [229–235]). These amines are oxidized to intermediates that coordinate tightly to the P450 ferrous heme, giving rise to a spectrum with an absorbance maximum at 445–455 nm [139]. Such MI-complex formation often, but not always, requires a primary amine moiety, but secondary and tertiary amines, as in the case of sertraline, amiodarone, TAO, and erythromycin, can also yield P450-MI complexes if they are first N-dealkylated to the primary or secondary amines by P450s or flavin -monooxygenases (FMOs; see below) [31, 228, 236].

Furthermore, unlike the competitive inhibition normally associated with type II coordination of amines to the P450 heme iron that occurs in the absence of any P450 catalytic turnover, the formation of these tight, quasi-irreversible MI complexes requires catalytic oxidation of the amine inhibitors (Fig. 5.7; [223, 226, 227]). Theprimary or secondary amines are apparently first hydroxylated, given that the corresponding hydroxylamines also generate similar complexes [138, 228, 236]. However, their coordination to the P450 heme iron requires a further oxidative step beyond the hydroxylamine [138, 226]. Indeed, the entity coordinating the heme iron is most likely the nitroso function obtained by a further two-electron oxidation of the hydroxylamine (Fig. 5.7; [138, 227, 237]). The ultimate oxidative step may not always require active P450 catalytic participation given the rather facile hydroxylamine autooxidation [238]. The coordination of a nitroso function is consistent with the finding that apparently identical complexes are obtained by reduction of nitro compounds [239]. The crystal structure of the MIcomplex of a model iron porphyrin with a nitroso compound indicates that the nitrogen rather than the oxygen of the nitroso group is the atom chelated to the iron [155].

Unlike the MDP-elicited MI complex discussed above, the alkyl/aryl amine -mediated P450 MI-complex can be easily disrupted upon oxidation of the P450-ferrous heme to the ferric state with potassium ferricyanide, with consequent reversion of the P450 enzyme to its native resting state. This oxidation-dependent reversal serves as a reliable diagnostic test of both alkyl- and aryl-nitroso MI complex es [231, 240]. On the other hand, in sharp contrast to the alkyl-nitroso

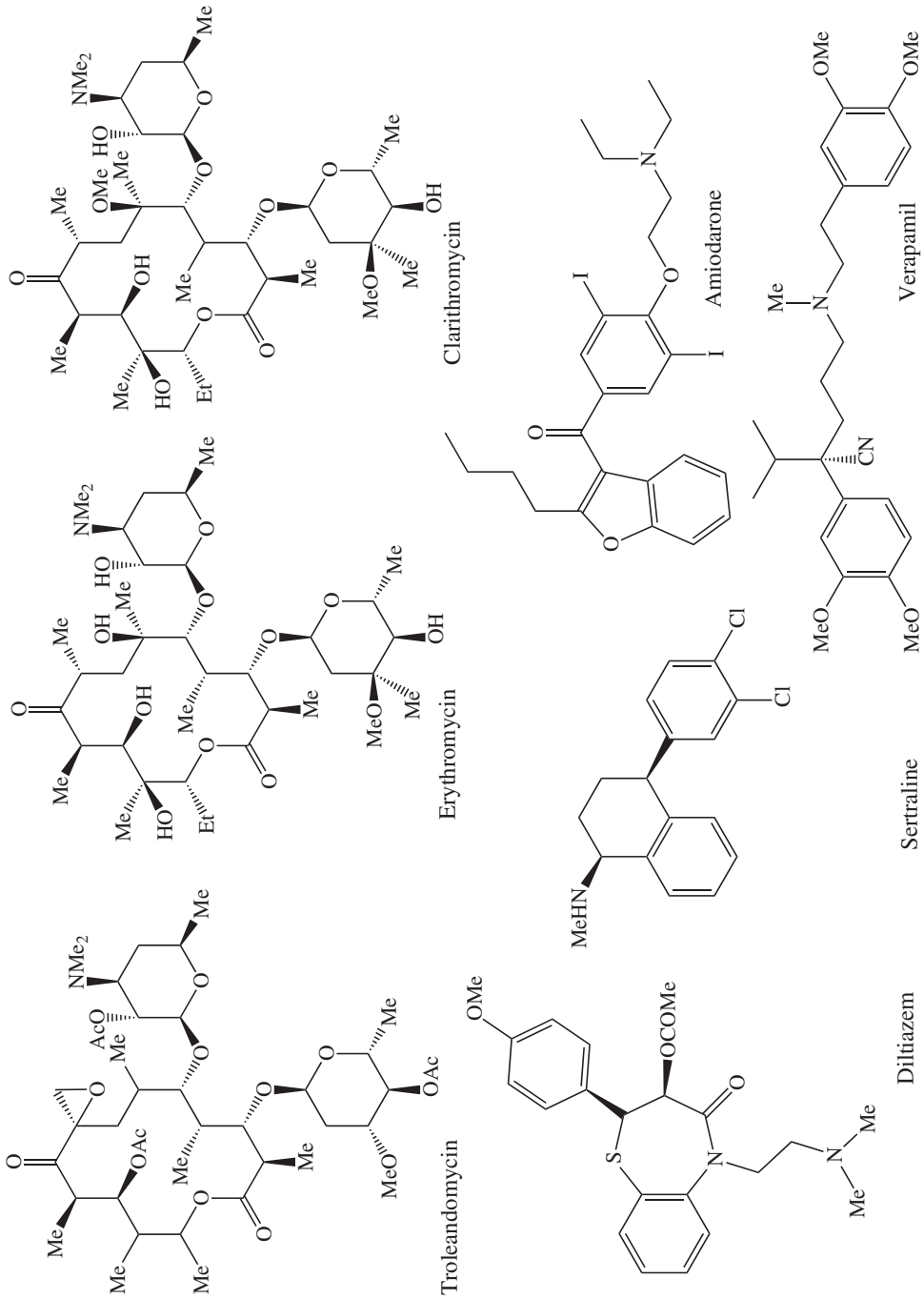


Fig. 5.6 Macrolide antibiotics and other therapeutic amine drugs documented to inactivate certain P450 isoforms via MIcomplexation. Following cointake of these therapeutic amines with other therapeutic drugs, in vivo P450 mechanism-based inactivations formed via either pathway illustrated in Fig. 5.7 result in clinically relevant DDIs. *MI* metabolic intermediate, *DDI* drug–drug interactions

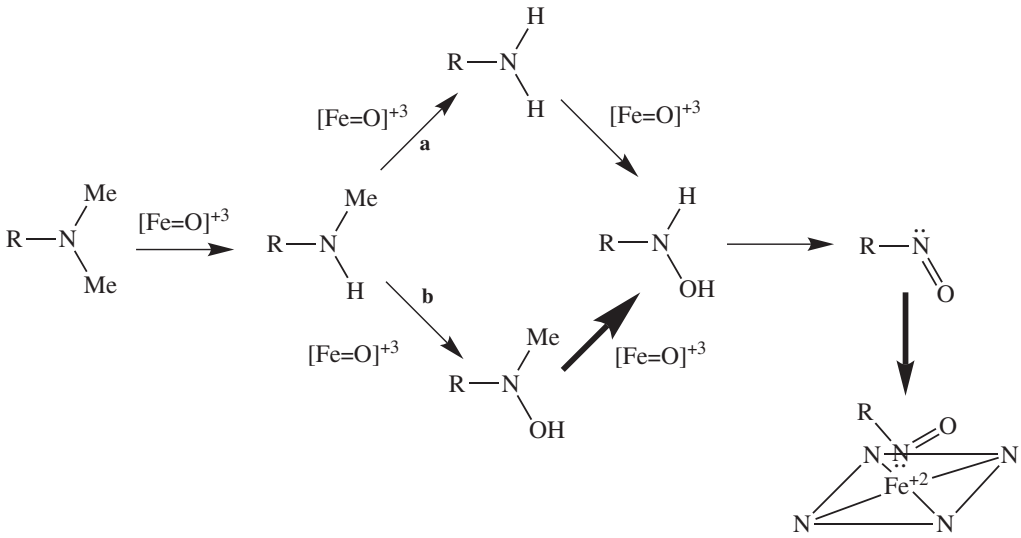


Fig. 5.7 Mechanistic pathways to the quasi-irreversible mechanism-based inactivation via P450-catalyzed MI-complex generation from amines. Pathway "a" entails P450-mediated sequential N-dealkylation of secondary and tertiary amines to the primary amine that is then oxidized to the hydroxylamine. The latter requires a further P450-mediated or autocatalytic oxidation to the nitroso function, whose nitrogen atom chelates the heme iron. Recent studies [228, 240] provide persuasive evidence that secondary and tertiary amines need not be first N-dealkylated to the primary amine, as secondary amine hydroxylamines are much more proficient in MI complexation than the corresponding primary amine derived hydroxylamines, thus favoring mechanistic pathway *b*

MI-complexes, the aryl-nitroso MIcomplexes are only transiently formed with NADPH- or dithionite-reduced microsomes [226]. They are unstable in the presence of excess dithionite, which reduces the nitrosomoiety back to the hydroxylamine [226]. The type II binding of this hydroxylamine to the ferrous P450 heme is apparently responsible for the observed spectral shift from 455 nm of the MI complex to the 423 nm peak of the type II complex on addition of excess dithionite [226]. Notably, this relative chemical instability of aryl-nitroso MI-complexes to either oxidation or reduction could most likely account for the observed underrepresentation of aryl amine drugs on the one hand, and the corresponding remarkable preponderance of alkyl amine drugs on the other, as MI complexed associated with a significant incidence of clinically relevant DDIs [31, 235, 236].

It is now increasingly evident that not all secondary amines require the initial N-dealkylation to the primary amine to form an MI complex (Fig. 5.7).The hydroxylamine metabolites of some secondary amines such as N-methylamphetamine [241], N-benzylamphetamine [242], desipramine, *S*-fluoxetine, N-desmethyldiltiazem, and sertraline are even more efficient MI complexes than the corresponding primary amines (Fig. 5.6; [228, 236, 243]). In the case of desipramine, *S*-fluoxetine, and *N*-desmethyldiltiazem, the relative rates of MI-complex formation have been shown to follow the order secondary hydroxylamine > secondary amine >> primary amine [236]. Furthermore, the ensuing primary amine metabolites were actually shown to competitively inhibit the P450 MI complexation by their corresponding precursor secondary amines, thereby indicating that *N*-hydroxylation rather than *N*-dealkylation of these secondary amines is the major pathway to their MI complexation [236]. This may also explain why in spite of higher circulating plasma levels of the primary alkyl amine metabolites relative to the parent drug, little correlation often exists between the *N*-dealkylation of some secondary alkyl amines by a given P450 enzyme and its MI complexation [31].

Moreover, while a secondary amine such as the SSRI sertraline is capable of being *N*-demethylated by multiple hepatic P450 isoforms (such as CYP2B6, CYP2C9, CYP2C19,

CYP2D6, and CYP3A4), only CYP3A4 incurs MBI, with K_I and k_{inact} values of 70.5 ± 14.4 μM and 0.131 ± 0.008 min^{-1}, respectively, yielding the characteristic 455-nm MI spectral signature upon incubation of HLMs with sertraline but not *N*-desmethylsertraline, its major *N*-demethylated metabolite [228]. These studies thus provide compelling additional support to the growing evidence that direct *N*-hydroxylation of the secondary amine rather than its *N*-demethylation to the primary amine is the critical pathway leading to its MI complex mediated MBI [228, 236].

They also underscore another relevant feature: Although multiple hepatic P450 enzymes metabolized the secondary amine sertraline to its primary amine (Fig. 5.7), they were not all susceptible to MBI by this drug [228]. This is also true of the *N*-demethylation of the SSRI fluoxetine to norfluoxetine by various human hepatic P450s including CYP2D6 and CYP2C19 [244]. In spite of its substantially higher affinity (K_m = 2.1 μM) and Cl_{int} = 2.9 μM^{-1} min^{-1} in this *N*-demethylation, CYP2D6 is not inactivated by the drug, whereas, CYP2C19 with an 82-fold lower affinity (K_m 172 μM) and Cl_{int} = 0.23 μM^{-1} min^{-1} incurs rapid MBI via MI-complex formation [31, 245, 246]. This differential susceptibility to MI complexation reveals that such differences may be dictated by the specific active site structural architecture of each P450 isoform. Thus, relative to various human P450 isoforms, the remarkably higher incidence of CYP3A4 MI complexation is attributed, at the least partly, to its spacious and highly promiscuous active site [31].

Accordingly, many clinically relevant alkyl amine drugs are known to inactivate CYP3A4 via MI complexation. For instance, in addition to inactivating CYP2C19, fluoxetine also inactivates CYP3A4 in a time- and concentration-dependent manner [246, 247], with a spectrally detectable (≈455 nm) MI complex most likely engendered via its hydroxylamine, with K_I and k_{inact} values of 5.26 ± 1.28 μM and 0.017 ± 0.002 min^{-1}, respectively, using CYP3A4-dependent midazolam 1′-hydroxylation as a functional probe [246, 247]. Similarly, the calcium channel blocker, verapamil (a tertiary alkyl amine; Fig. 5.6) and its major metabolites *N*-desalkylverapamil and norverapamil are all found to inactivate CYP3A4, yielding the spectral MI-complex signatures with K_I and k_{inact} values of 6.46 ± 2.19 μM and 0.39 ± 0.06 min^{-1} for *R*-verapamil, 2.97 ± 0.30 μM and 0.64 ± 0.04 min^{-1} for *S*-verapamil, 5.89 ± 0.83 μM and 1.12 ± 0.08 min^{-1} for (±) norverapamil, and 7.93 ± 0.45 μM and 0.07 ± 0.00 min^{-1} for *N*-desalkylverapamil with the functionally reconstituted recombinant CYP3A4 enzyme [248–250]. The CYP3A4 inactivation potency of the verapamil enantiomers and their metabolites based on their individual k_{inact}/K_I ratios could be ranked in the order: *S*-norverapamil > *S*-verapamil > *R*-norverapamil > *R*-verapamil > *N*-desalkylverapamil [249, 250]. Interestingly, these studies also revealed that, although the secondary alkylamine *N*-desalkylverapamil was found at plasma levels comparable to those of the parent drug and its *N*-demethylated metabolite norverapamil (another secondary alkylamine), it was not quite as efficient as either of the latter two in CYP3A4 MBI. This could partly be due to its higher K_I value [249, 250], possibly reflecting less tighter binding to the CYP3A4 active site upon loss of the lipophilic 2-(3,4-dimethoxyphenyl)-ethyl moiety through *N*-dealkylation.

Administration of the antiarrhythmic tertiary amine amiodarone (Fig. 5.6) to rodents (rats, mice, and hamsters) is also known to result in MI complexes, most likely derived from a nitroso metabolite [251]. Although in vivo amiodarone is known to interact with substrates of human CYP1A2, CYP2C9, CYP2D6, and CYP3A4, its MBI potential in vitro towards each of these P450s is somewhat ambiguous. Thus, both amiodarone and its major metabolite *N*-desethylamiodarone are capable of inactivating P450s, but in some cases are found to do so differentially. In one study [252] with recombinantly expressed P450s and diagnostic functional probes, amiodarone but not *N*-desethylamiodarone, inactivated CYP3A4 with K_I and k_{inact} values of 13.4 μM and 0.06 min^{-1}. *N*-desethylamiodarone, in the other study [253], inactivated various other P450s with respective K_I and k_{inact} values of 1.0 μM and 0.03 min^{-1} for CYP1A1, 11.6 μM and 0.03 min^{-1} for CYP1A2, 0.6 μM and 0.02 min^{-1} for CYP2B6, and 1.3 μM and 0.12 min^{-1} for CYP2D6. In yet another study [254], using the cocktail substrate mixture approach, both the

parent drug and its major metabolite inactivated CYP3A4 in a time- and concentration-dependent manner with K_I and k_{inact} values of 42.4 μM and 0.02 min^{-1} for amiodarone, and 25.8 μM and 0.03 min^{-1} for *N*-desethylamiodarone, the latter exhibiting a greater inactivation potency (k_{inact}/K_I ratio of 1.24:0.52 for the parent drug). This study found that both amiodarone and *N*-desethylamiodarone also inactivated CYP2C9, but that only *N*-desethylamiodarone inactivated CYP2D6 (K_I and k_{inact} values of 29.8 μM and 0.05 min^{-1}), rather potently (k_{inact}/K_I ratio of 1.61) [254].

The consistent finding that only *N*-desethylamiodarone, but not amiodarone, inactivates CYP2D6 raises another issue: Given that several P450 isoforms metabolize amiodarone to *N*-desethylamiodarone, the possibility exists that in vivo, the reactive metabolite generated by one P450, on escape from its active site may actually inactivate another susceptible neighboring P450, even though the latter is not itself directly responsible for the initial *N*-dealkylation in the inactivation cascade. In the case of secondary and tertiary amines, the principal instigator of the MBI may even be a non-P450 enzyme such as a flavin-containing monooxygenase (FMO). Accordingly, it was shown that *N*-cyclopropylbenzylamine forms MI complexes with P450s in liver microsomes, but not in liver microsomes gently preheated so as to inactivate FMOs, or in functionally reconstituted systems that excluded FMOs [255]. By contrast, *N*-hydroxy-*N*-cyclopropylbenzylamine and *N*-benzylhydroxylamine were much more efficient at generating MI complexes in liver microsomes gently preheated so as to inactivate FMOs or functionally reconstituted systems [255]. The corresponding nitrone (PhCH = N(O)cPr) species is even more efficient than the parent compound, and such inactivation is considerably much faster than the hydrolysis of *N*-hydroxy-*N*-cyclopropylbenzylamine to a primary hydroxylamine. Based on these findings, the proposed reaction trajectory to the MI complex entails an initial oxidation of *N*-cyclopropylbenzylamine by a microsomal FMO to *N*-hydroxy-*N*-cyclopropylbenzylamine, which is further oxidized either by a P450 (CYP2C11) or a FMO to a different nitrone (C_2H_4C=N(O)CH_2Ph) which hydrolyzes to *N*-benzylhydroxylamine and is further oxidized to yield the nitroso –MI complex or the oxime product [255]. These findings underscore the strong possibility that the potential for in vivo MI-complex formation and consequent clinically relevant DDIs of a drug candidate may be seriously overlooked, if its MBI potential were to be assessed in vitro with just one P450 or one functional probe. Apparently, multiple other P450s and/or FMOs may participate in generating suitable metabolic intermediates that serve as reactive precursors for MI complexation of certain P450s.

The tertiary amine macrolide antibiotic s erythromycin and troleandomycin (TAO; Fig. 5.6) have long been known to form typical 455-nm MI complex es, particularly with CYP3A enzymes in humans as well as rodents [140, 142, 224, 225, 229, 230, 256, 257]. The MI complexes can be isolated intact and purified, and at a time when recombinant P450 technology was not yet available, such MI complexation afforded a convenient approach to purify the relatively intractable CYP3A enzymes from liver microsomes [140, 258, 259]. Such in vivo MI complexation of TAO or erythromycin to the heme of CYP3A enzymes not only inhibits their functional activity but also stabilizes them and prolongs their half-lives in hepatocytes [142]. Functional dissociation of these MI complexes with ferricyanide is found to fully restore their activity. A major consequence of such in vivo MI complexation, particularly on repeated administration, is that the cellular CYP3A protein is increased, due to "induction" via protein stabilization [142, 257–259]. This CYP3A protein stabilization could stem from substrate-induced conformational stabilization and/or suppression of its futile oxidative turnover in vivo by MI complexation. This latter possibility is the most plausible, given that inhibition of CPR [10, 11] or conditional deletion of CPR [12, 13] that suppresses catalytic turnover, also similarly results in P450 induction via stabilization.

The broad-spectrum macrolide antibiotic clarithromycin (Fig. 5.6) has been shown to form hepatic CYP3A –MI complexes when administered to control or dexamethasone (DEX)-pretreated rats [229, 260]. In vitro studies with DEX-pretreated rat liver microsomes (enriched in CYP3A

content) revealed that MI complexation was most efficient with clarithromycin *N*-oxide and *N*-desmethylclarithromycin relative to the parent compound both in their time of onset as well as extent. Repeated intraperitoneal administration of these compounds to rats also revealed that these two metabolites were more efficient than the parent compound in "inducing" hepatic P450 content, albeit not quite as potently as TAO [260]. Biopsy sampling of the duodenal mucosa of human volunteers repeatedly administered clarithromycin (500 mg twice daily/7 d) revealed that it also reduced their duodenal CYP3A-dependent 1′-hydroxymidazolam and 4-hydroxymidazolam hydroxylation by 74and 63 %, respectively, versus the corresponding baseline values [261]. This clarithromycin-elicited lowering of the intestinal CYP3A content was associated with a doubling of the dose-normalized midazolam plasma concentration after intravenous administration and a corresponding decrease in the ratio of serum 1′-hydroxymidazolam/midazolam relative to baseline values, consistent with the observed doubling of the gut wall bioavailability of oral midazolam [247]. Immunoblotting analyses revealed a small, albeit not statistically significant increase in intestinal CYP3A4/5 content normalized to villin content, consistent with possible CYP3A stabilization upon clarithromycin-elicited MI complexation [261]. Furthermore, individuals exhibiting overall higher CYP3A activity due to expression of both CYP3A4 and functionally active CYP3A5 were proposed to be at a greater risk for clarithromycin-elicited DDIs than individuals lacking functional CYP3A5 expression [261]. The macrolide antibiotic tiamulin, a semisynthetic derivative of the antibiotic pleuromutilin, is used in meat producing domestic animals in Europe and Mediterranean countries for the treatment of enteric and respiratory diseases, and such use in veterinary medicine is associated with toxic DDIs, when coadministered with other P450 competitive drug substrates or inhibitors. Indeed, tiamulin was also found to generate MI complexes with CYP3A enriched rifampin-pretreated rabbit liver microsomes [262]. At 125-μM concentration, the extent of CYP3A MI complexation was in the order of tiamulin>erythromycin>TAO>roxithromycin>tylosin, but non-detectable with spiramycin and tylmicosin [262].

Finally, HIV-protease inhibitors such as amprenavir, indinavir, nelfinavir, lopinavir, saquinavir, and RTV have long been known as potent P450 inhibitors [263–270]. When their MBI potential was tested using pooled HLMs, recombinant rCYP3A4 (+b_5), and rCYP3A5 (+b_5), with CYP3A -dependent testosterone 6β-hydroxylation as the functional probe [31, 271], all these agents exhibited time- and concentrationdependent MBI, with RTV (See Fig. 5.15) being not only the most potent (K_I=0.10 and 0.17 μM, respectively), but also the most efficient (k_{inact}=0.32 and 0.42 min^{-1}, respectively) against rCYP3A4 (+b_5) and HLMs [271]. On other hand, nelfinavir was the most efficient inactivator of CYP3A5 (k_{inact}=0.47 min^{-1}) and RTV, the most potent (K_I=0.12 μM). Most importantly, all of these HIV-protease inhibitors with the exception of lopinavir and saquinavir were also found to exhibit spectrally detectable MI complexation of rCYP3A4 (+b_5) [271]. This is particularly intriguing for RTV, whose potential for MI complexation is not entirely obvious from either its chemical structure or its major site of oxidation (See Fig. 5.15). While the role of RTV as a potent CYP3A inhibitor is incontrovertible and supported by ample in vivo data, the mode of this inhibition remains highly controversial. To date, the mechanisms of its CYP3A inhibition include: Irreversible type II binding with consequent lowering of the P450-heme redox potential to effectively block CPR-electron transfer [272], MBI via MI complexation [271], and MBI via heme-adduction to the CYP3A protein[265, 273; see below]. Although supportive experimental evidence exists for each of these mechanisms, it is puzzling how the virtual catalytic blockade invoked in the first mechanism could ever be reconciled with the other two inactivation modes that require P450 catalytic turnover and thus CPR-reduction.

5.3.1.3 1,1-Disubstituted and Acyl Hydrazines

A similar mechanism is also involved in P450 inhibition by 1,1-disubstituted hydrazines and acyl

hydrazines. In this process, 1,1-disubstituted, but not monosubstituted, hydrazines are oxidized by P450 enzymes to products that coordinate tightly to the heme-iron atom (24). These complexes are also formed in a time-, NADPH-, and oxygen-dependent manner [274], and are characterized by a ferric absorption maximum at ~438 nm and a ferrous absorption maximum at 449 nm [274]. Liver microsomal P450s oxidize isoniazid and other acyl hydrazines, yielding a transient complex with a similar absorption maximum at 449 nm [275, 276]. However, the isoniazid complex is only stable in the ferrous state, dissociating upon the addition of ferricyanide [277]. Model studies with synthetic iron-porphyrins indicate that 1,1-dialkylhydrazines are oxidized to disubstituted nitrenes that form end-on complexes with the iron. The nitrene complexes formed in the reactions of 1-amino-2,2,6,6-tetramethylpiperidine and several iron tetraarylporphyrins, characterized by nuclear magnetic resonance (NMR), Mössbauer, and X-ray analyses [278, 279], strongly support the likelihood that the P450 complexes generated during the oxidation of 1,1-disubstituted hydrazines, and possibly acyl hydrazines, are aminonitrene-iron complexes (Fig. 5.8). This oxidative conversion of the dialkylhydrazines to aminonitrenes could occur either via initial hydroxylation of the hydrazine or via stepwise electron removal from the hydrazine (Fig. 5.8).

5.3.2 Covalent Binding to the Prosthetic Heme and Modification of the P450 Protein by Heme Fragments

P450s may often be irreversibly inactivated via reaction of the reactive intermediate formed from the mechanism-based inactivator by covalent modification of the heme group [24]. In numerous instances, heme alkylation has been demonstrated by the isolation and structural characterization of modified hemes [24]. Structural characterization of the modified hemes is essential since loss of enzyme content that is comparable to loss of heme does not always establish unambiguously that heme modification is responsible for enzyme inactivation. It is also possible that a heme adduct is formed that is either reversible or too unstable to be isolated. The quantitative correlation of enzyme inactivation with the formation of a heme adduct(s) is technically quite difficult. In the absence of such data, it is difficult to rule out the possibility that the enzyme is inactivated in part by mechanisms such as protein modification even when it has been conclusively demonstrated that there is heme alkylation.

In the third edition of *Cytochrome P450: Structure, Mechanism, and Biochemistry* (Chap. 7), the processes of the covalent binding of mechanism-based inactivators to the prosthetic heme, the modification of the P450 apoprotein by

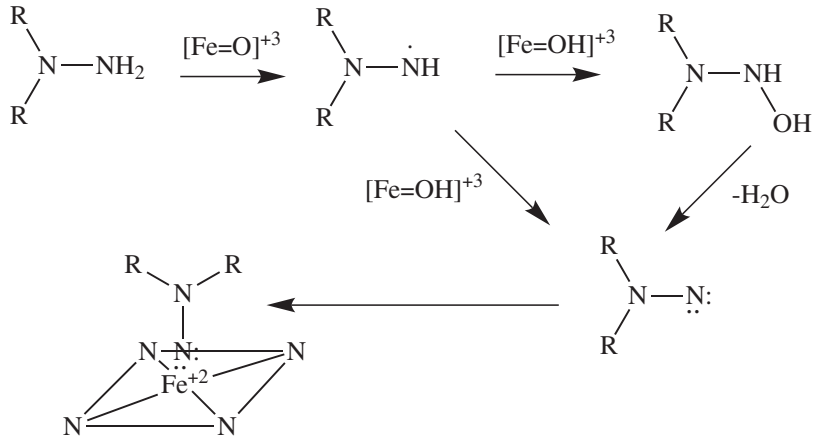


Fig. 5.8 Mechanistic pathway for P450 heme iron coordination by the nitrene species derived from the metabolic activation of 1,1-dialkylhydrazines

heme fragments, as well as other modes of P450 heme degradation brought about by mechanism-based inactivators are discussed in detail [24]. The reader is referred to that very comprehensive review for more details. It is noteworthy, however, that these processes can have significant cellular and pharmacological consequences. Accordingly, the heme-modified P450 proteins are sensed as "aberrant/damaged" and targeted for cellular disposal by the ubiquitindependent 26S proteasomal degradation [280]. Furthermore, as discussed elsewhere [14], the heme-denuded P450 proteins may also be subject to increased proteolytic disposal, if the supply of fresh cellular heme required for heme recycling upon destruction of the existing prosthetic heme moiety is inadequate.

5.3.3 Covalent Binding to the P450 Protein

A variety of functional groups that either occur naturally or have been specifically engineered into drugs to increase their stability, solubility, or bioavailability have been shown to predispose these drugs to metabolism by a particular P450 isozyme or isozymes in such a way as to generate reactive intermediates that can lead to MBI by one or more of the previously mentioned routes. These molecules can be grouped according to their structural aspects and they fall into a number of major categories including: (a) various sulfur-containing compounds (e.g., carbon disulfide [281–283], diethyldithiocarbamate [284], isothiocyanates [285], mercaptosteroids [286–293], parathion [294,295], thioureas [296], thiophenes [297], and tienilic acid [298]; (b) various halogen containing compounds such as chloramphenicol [299–302], *N*-monosubstituted dichloroacetamides [303], and *N*(2-*p*-nitrophenethyl) dichloroacetamide [304]; (c) acetylenes and alkyl and aryl olefins [305–311] such as 10-undecynoic acid [305,310], 1-ethynylpyrene [308,310], 17β-ethynylprogesterone [312,313], 17α-ethynylestradiol [313–318], 1- and 2-ethynylnaphthalene [306,307,309,319], 7-ethynylcoumarin (7-EC) [320], gestodene [321], mifepristone [322,323], and secobarbital [324]; (d) furanocoumarins such as bergamottin and 6′,7′-dihydroxybergamottin (6′,7′-DHB) [325–327], 8-methoxypsoralen (8-MOP, methoxsalen) [328–336], and the furanopyridine, L-754,394 [337–339] and (e) compounds such as carbamazepine (CBZ) and tamoxifen that are hydroxylated to form catechol metabolites [340–343]. Although the details of the mechanisms by which some of these compounds inactivate the P450s remain unclear, we now have significant information about the mechanisms by which many of these inhibitors are activated to form reactive intermediates and, in some cases, information is known about the sites on the P450s where they bind covalently leading to the inactivation.

5.3.3.1 Organosulfur and Halogenated Compounds

The incubation of liver microsomes with [^{35}S]-parathion leads to radiolabeling of the apoprotein; however, there is no radiolabeling of the protein when the parathion ethyl groups are ^{14}C-labeled [294,295]. Immunoprecipitation of the labeled P450 using anti-P450 antibodies leads to recovery of 90% of the ^{35}S-label covalently bound to microsomal proteins. During the incubation, approximately 75% of the P450 prosthetic heme is degraded to unknown products, but ~4 nmol of radiolabeled sulfur are covalently bound to the apoprotein for each nmol of heme chromophore that is lost. Most (50–75%) of the radiolabeled sulfur can be removed from the protein by treatment with dithiothreitol (DTT) or cyanide, suggesting that the bulk of the sulfur label is present in the form of hydrodisulfides (RSSH). However, the enzyme cannot be reactivated by these treatments. The binding of multiple equivalents of radiolabeled sulfur to the apoprotein in these studies suggests that catalytic activation of the sulfur of parathion continues despite covalent attachment of the sulfur to the protein until the residue on the protein that is critically involved in catalysis is modified or the heme itself is damaged or is released from the protein as a consequence of multiple reactions damaging the apoprotein [294]. A suggested mechanism for the inactivation is shown in Fig. 5.9. This mechanism is sup-

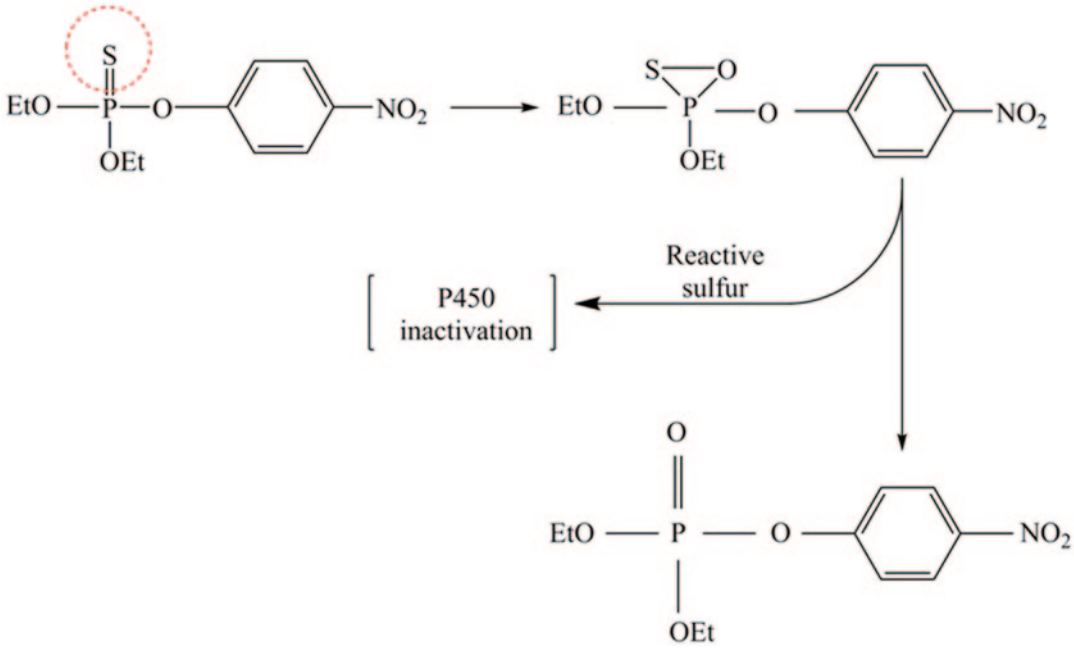


Fig. 5.9 The activation of parathion to form a reactive intermediate that causes mechanism-based inactivation. This is thought to involve formation of the putative reactive phosphooxythiran intermediate that is then responsible for the protein modification. The circled area indicates the site of metabolism

ported by the following observations: (a) covalent binding of the radiolabeled sulfur to the apoprotein; (b) the ability of P450s to oxidize sulfur compounds to *S*-oxides; and (c) the formation of metabolites where oxygen has replaced the sulfur. Studies by Murray and coworkers have demonstrated that at low concentrations parathion competitively inhibits some rat liver P450s (i.e., CYPs 2B1 and 2C6), whereas at higher concentrations it inactivates several P450s, including CYPs 2A1, 2A2, 2C11, 3A2, and 3A4, but does not inhibit CYP2B1 or CYP2D6 [344–347]. The inactivations are observed in vitro but not in vivo [345–348]. Studies with human liver microsomes in vitro demonstrated that CYP3A4 is the principal isoform inactivated in human liver microsomes, whereas CYPs 2C9 and 1A2 are minor forms that are also inactivated, whereas there is no inactivation of CYP2E1 [344–347]. Although incubation of parathion with NADPH-supplemented rat and human liver microsomes or purified recombinant P450 s leads to destruction of the prosthetic heme, the relevance of these findings in vivo has not yet been established [348]. The concentrations of parathion required for MBI of the P450s in vitro are considerably higher than the concentrations causing death through their inhibition of acetylcholinesterase [348, 349].

Recent studies using rat liver microsomes have demonstrated MBI of the P450s involved in the desulfuration of methyl parathion. Incubation of the microsomes with methyl parathion resulted in a 58% decrease in the spectrally observed P450 content [350]. This loss of activity was not associated with a comparable increase in the absorbance at 420 nm in the difference spectrum, suggesting that the heme had been displaced from the apoprotein. Since the rates for the metabolism of testosterone to form the 2β- and 6β-hydroxy products were reduced to 8 and 2%, respectively, the authors concluded that CYP3A4 and CYP211 were the P450s inactivated during the oxidative desulfuration of methyl parathion [350]. The modified P450s from the liver microsomes of male rats following incubation with methyl parathion were resolved by so-

dium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and then digested with trypsin and the peptides were analyzed by nanospray tandem mass spectrometry. Several peptides were identified exhibiting increased masses of 96 amu due to the formation of sulfur adducts. These peptides were sequenced and the adducts were shown to be on cysteines 64 and 378 of CYP3A1. A CYP3A1 homology model based on the human CYP3A4 crystal structure suggested that these two cysteines are not located in the enzyme catalytic site, but appear to be located near the surface of the protein along a channel through which the substrate gains access to the active site. Therefore, it was suggested that these modifications might hinder substrate entry into the active site binding pocket or coordination with that site [350].

A number of different substituted thiophenes have been shown to be mechanism-based inactivators of P450s. Tienilic acid (Fig. 5.10), a substituted thiophene diuretic was withdrawn from the market because of its liver and kidney toxicity. Tienilic acid is metabolized by human liver CYP2C9 to yield 5-hydroxy tienilic acid. During the metabolism of tienilic acid, a highly reactive electrophile is generated that covalently binds to the CYP2C protein leading to the inactivation of the enzyme [297, 349, 351]. Although covalent labeling of the protein is partially prevented by glutathione (GSH), GSH does not protect the enzyme from inactivation or protein modification. In the presence of GSH, the ratio of label to protein is approximately 0.9 following inactivation. These results have been explained by the formation of a sulfoxide of the thiophene that then can react with water to give the 5-hydroxy tienilic acid, with a protein nucleophile to inactivate the enzyme, or with GSH after leaving the P450 active site. The identity of the amino acid residue modified by tienilic acid has not yet been determined, but high-performance liquid chromatography (HPLC)/electrospray ionization mass spectrometric analysis (ESI-MS) of the modified and native proteins reveals the presence of CYP2C9 modified proteins with molecular masses of 55,923 ± 1.1 and 56,273 ± 4.4 Da, which correspond to mass shifts of 344.4 ± 1.1 and 694 ± 4.2 Da, respectively, suggesting that the proteins have been modified by both the for-

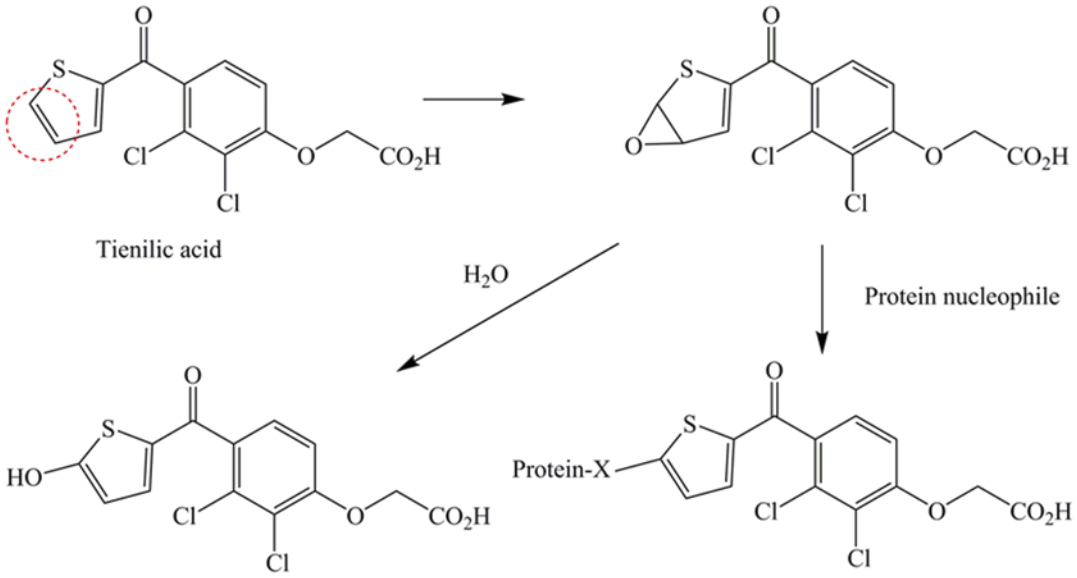
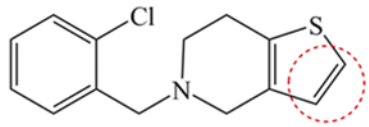


Fig. 5.10 Tienilic acid is thought to be activated on the thiophene ring to an epoxide intermediate that can then react either with a P450 nucleophilic residue, resulting in mechanism-based inactivation, or be hydrolyzed to give the thiophene ring hydroxyl product. The circled area indicates the site of metabolism leading to the reactive epoxide

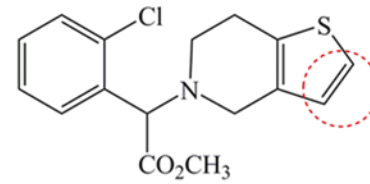
mation of a single and two simultaneous adducts. The inclusion of GSH (10 mM) in the incubation mixture abolishes the formation of the adducted protein, suggesting that the second adduct may result from modification of a residue that is outside of the active site. The mass shift of 344.4 ± 1.1 is consistent with the binding of one molecule of monohydroxylated tienilic acid which may be formed either by ring oxidation of the thiophene or by formation of a sulfoxide that does not undergo dehydration [349].

3-[(Quinolin-4-ylmethyl)-amino]-*N*-[(4-trifluoromethox)phenyl]thiophene-2-carboxamide (OSI-930), an investigational anticancer agent containing a thiophene moiety (Fig. 5.11), inactivated CYP3A4 in a mechanism-based manner [352]. Spectral analysis indicated that the decrease in the reduced CO-spectrum at 450 nm was equal to the amount of inactivation suggesting that the inactivation was primarily due to the modification of the heme. Since OSI-930 has no effect on CYP3A5 activity, it suggests that this may be an excellent compound for distinguishing between the relative clearance roles of these two structurally similar enzymes, and may be of great value in exploring the unique aspects of their very tolerant and overlapping substrate-binding active site s [352].

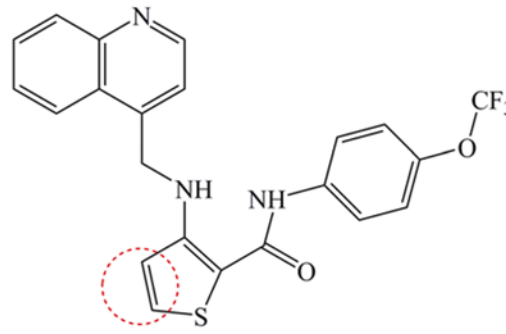
Spironolactone (Fig. 5.12) is an antagonist of aldosterone which is used as a diuretic and antihypertensive [353]. Spironolactone inactivates P450s in both hepatic and steroidogenic tissues [286–293], including members of the hepatic CYP2C and CYP3A subfamilies [289, 290], as well as adrenal CYP17A1 [286, 287, 292, 293]. The spironolactone-mediated inactivation of CYP2C and CYP3A requires hydrolysis of the thioester group to give the free thiol that is then oxidized to give a reactive intermediate that can form adducts with either the protein and/or the heme [288, 289]. The inactivation of CYP17A1 appears to result from the thiosteroid binding covalently to an amino acid residue(s) on the protein. Enzyme inactivation as a consequence



Ticlopidine



Clopidogrel



OSI-930

Fig. 5.11 The structures of three other thiophene-containing drugs. Ticlopidine, clopidogrel, and OSI-930 may be activated by epoxide formation on the thiophene ring as shown in Fig. 5.10. The circled areas indicate the postulated sites of epoxidation

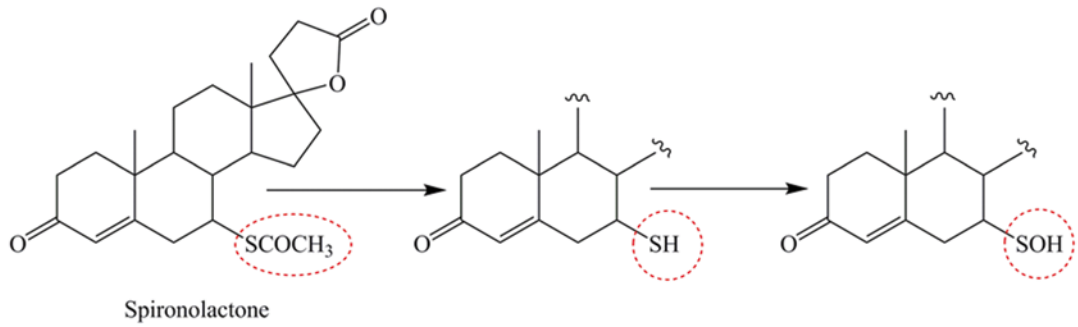


Fig. 5.12 Spironolactone bioactivation. The sequence of steps for the activation of spironolactone to a reactive intermediate that can inactivate CYP17A1 by covalent modification of the protein and can also inactivate hepatic CYPs 3A by destruction of the heme group to reactive fragments that irreversibly modify the protein. The first step involves thioesterase-catalyzed hydrolysis of the thiol ester to yield a thiol group, which then can be oxidized by the P450 to a reactive intermediate that serves as the ultimate mechanism-based inactivator. As described in the text, the sulphydryl group is oxidized to a species that then can react with either the protein or the heme. The circled areas indicate the sites of metabolism leading to the ultimate reactive intermediate causing inactivation

of oxidation of the thiol group is suggested by the observation that in rat hepatic microsomes in which CYPs 3A have been induced, these P450s are thought to oxidize the thiol group (SH) to the sulfinic (-SO_2H) and sulfonic (-SO_3H) acids [289], ultimately giving rise to a disulfide adduct with GSH [293]. Formation of this GSH disulfide adduct appears to be catalyzed, at least in part, by a flavin monooxygenase [293]. Oxidation of the thiol group may lead to the formation of either a sulfhydryl radical (-S°) or the sulfenic acid (-SOH), either one of which, or both, may be involved in the P450 inactivation. The reaction of the sulfhydryl radical may lead to fragmentation of the heme with consequent protein modification, whereas the reaction of the sulfenic acid with an amino acid side chain may lead to protein modification (Fig. 5.12).

Derivatives of thiazolidinedione (TZD) such as MK-0767 or troglitazone (TGZ, rezulin; Fig. 5.13) have been shown to undergo metabolism by P450s via activation of the TZD ring followed by ring scission to generate several reactive intermediates [353, 354]. TGZ was the first oral glitazone used successfully for the treatment of type II diabetes [354]. In 2000, it was voluntarily withdrawn from the market due to its association with severe hepatotoxicity that led to approximately 90 cases of hepatic failure requiring liver transplantation as well as 26 deaths [353]. TGZ is metabolized primarily by CYP3A4 and is also a potent inducer of that P450. Primary human metabolism involves sulfation to form the TGZ-sulfate (TGZS), oxidative opening of the chroman ring to give a TGZ-quinone (TGZQ), and glucuronidation to yield the TGZG product. Covalent binding of [^{14}C]TGZ to macromolecules was primarily seen in rat liver microsomal preparations from DEX-induced rats, suggesting a role for CYPs 3A. Covalent binding was NADPH-dependent and was completely inhibited by the addition of ketoconazole, suggesting a requirement for a functionally active P450. Although TGZ hepatotoxicity is currently thought to arise through a variety of mechanisms, reactive intermediates of TGZ such as the epoxide or the quinone may play important roles in many of its pathological consequences.

Raloxifene (Fig. 5.13) is a selective estrogen receptor modulating drug (SERM) that has been used for the treatment of postmenopausal osteoporosis [355]. MBI of human liver microsomal CYP3A4 is observed during the metabolism of raloxifene .CYP3A4 activated raloxifene primarily by metabolism at the seven-position and to a lesser extent at the five-position of the benzothiophene ring, as well as at the three-position of the phenol ring. It was suggested that the mecha-

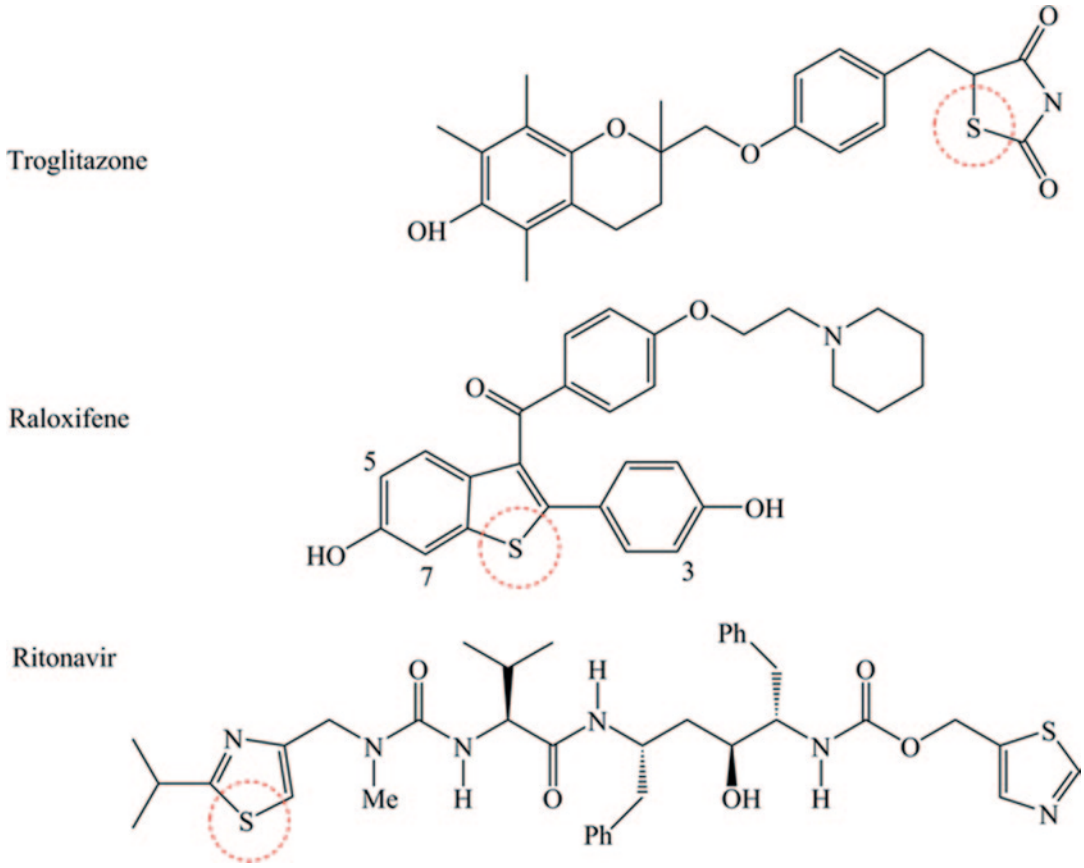


Fig. 5.13 Structures of three sulfur-containing compounds known to serve as mechanism-based inactivators of several P450s by mechanisms that have not yet been elucidated, but in all probability involve oxidation of the sulfur atom. The circled areas indicate the sites of the sulfur oxidation that may be involved in the inactivation reaction

nism involved initial epoxidation of the phenol to form a reactive arene oxide intermediate [355]. However, the possibility of a quinone intermediate could not be ruled out. Liquid chromatograpy (LC)–mass spectrometry (MS) studies demonstrated that a single equivalent of raloxifene was bound to the intact apoprotein. Mass analysis of peptides following proteinase K digestion of the inactivated protein revealed that raloxifene was adducted to the Cys239 residue. LC–MS analysis of the intact protein revealed a mass shift of 471 Da for the inactivated protein relative to controls, indicating that the inactivation occurred through the formation of a raloxifene diquinone methide that underwent nucleophilic attack by the Cys239 sulfhydryl. Based on the formation of GSH adducts, it has been demonstrated that raloxifene is also bioactivated by a number of other P450s, including CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A5 [356]. Although all of these P450s catalyzed the bioactivation, only CYP2C8 and CYP3A4 exhibited raloxifene-mediated MBI [356]. The inactivation kinetics were relatively comparable with K_i and k_{inact} values of 0.26 μM and 0.10 min^{-1} and 0.81 μM and 0.20 min^{-1} for CYP2C8 and CYP3A4, respectively. Tryptic digestion followed by LC–MS analysis of the tryptic peptides revealed the formation of adducts to Cys239 and Cys225 of CYP3A4 and CYP2C8, respectively. For each of the P450 isozymes that catalyzed the bioactivation of raloxifene, possible access/

egress channels for the substrate/metabolites were mapped and only CYP3A4 and CYP2C8 were shown to possess accessible cysteine residues near the active site cavities. This result is consistent with the observation that these two forms of P450 were the only ones inactivated by raloxifene. These results suggest that bioactivation of a given substrate to a reactive intermediate is necessary for MBI of that P450, but that it is not sufficient, and that the extent of bioactivation does not necessarily correlate with the extent of MBI of the P450 that bioactivates it. Thus, it is clear that multiple factors contribute to the ability of reactive metabolites to form adducts with P450s leading to MBI. Except for CYP2E1, each of the P450s investigated formed the diquinone methide of raloxifene, which then reacts with GSH to form a GSH adduct. In CYP3A4 and CYP2C8, the presence of a cysteine residue in the active site that could be alkylated following the formation of the diquinone methide was essential for inactivation of the enzyme. The lack of inactivation of CYP1A2, CYP2D6, and CYP3A5 is consistent with crystal structure data that show there are no cysteines present in the vicinity of their active sites [356]. These results suggest that there is no correlation between the extent of reactive metabolite formation by a P450 and its inactivation. Thus multiple additional factors including the architecture of the active site, the lack or presence of appropriate nucleophilic residues in the exit channel, and the reactivity and structure of the reactive metabolite may all contribute to the ability of a P450 to be inactivated during metabolism of a compound that forms reactive intermediates and that could lead to the formation of a protein adduct.

Ritonavir (RTV; Fig. 5.13) has been shown to be a potent reversible inhibitor as well as a mechanism-based inactivator of CYP3A4/CYP3A5 [263]. RTV is currently used at low doses in combination with other protease inhibitors such as saquinavir, amprenavir, and lopinavir in order to "pharmacologically boost" the bioavailability of the other protease inhibitors by inactivating or inhibiting CYP3A4 [265]. Its inhibitory potency for CYP3A4 is dependent on the presence of both the 2-(1-methylethyl)thiazolyl group as well as the 5-thiazolyl group. It is believed to be oxidized to a chemically reactive intermediate containing the 2-(1-methylethyl)thiazolyl group that is responsible for P450 inactivation [265]. RTV has also been shown to be a mechanism-based inactivator of human CYP2B6 in a functionally reconstituted system [273]. CYP2B6 inactivation by RTV is time-, concentration-, and NADPH-dependent with a K_I of 0.9 μM, a k_{inact} of 0.05 min^{-1}, and a partition ratio of approximately 3. Liquid chromatography-tandem mass spectrometry (LC–MS/MS) revealed two major metabolites, an oxidation product and a deacylated product [273]. MBI of CYP2B6 resulted in a loss of native heme comparable to the loss of its activity with no modification of the apoprotein observed by LC–MS. RTV was also found to be a potent mechanism-based inactivator of CYP3A4 and the molecular mechanism involves heme destruction with the formation of a heme-protein adduct [273]. Similar to CYP2B6, no significant modification of the apoprotein was observed. LC–MS/MS analysis of the incubation mixture resulted in the identification of an RTV–glutathione conjugate having an MH^+at M/Z 858, suggesting that the formation of an isocyanate intermediate was responsible for the formation of the conjugate [273].

The isothiocyanates (ITCs) are found as glucosinolate complexes and are in great abundance in various cruciferous vegetables such as cabbage, broccoli, and watercress [357]. The effectiveness of the ITCs as mechanism-based inactivators is based on the reactivity of the electrophilic carbon center with sulfur, nitrogen-, or oxygen-containing nucleophilic residues in the P450. Many naturally occurring and synthetic ITCs inhibit the activities of a variety of different P450 isozymes including CYP2A6/13, CYP2B1/6, and CYP2E1 in vivo and in vitro [358, 359]. The inactivation is thought to occur either by a direct interaction of the ITC with one or more nucleophilic residues on the apoprotein or by a metabolic activation of the ITC to a reactive intermediate that then forms a covalent adduct, thereby inactivating the P450 (Fig. 5.14). Benzyl isothiocyanate (BITC) and phenethyl isothiocyanate (PEITC), two naturally occurring isothiocyanates, were shown

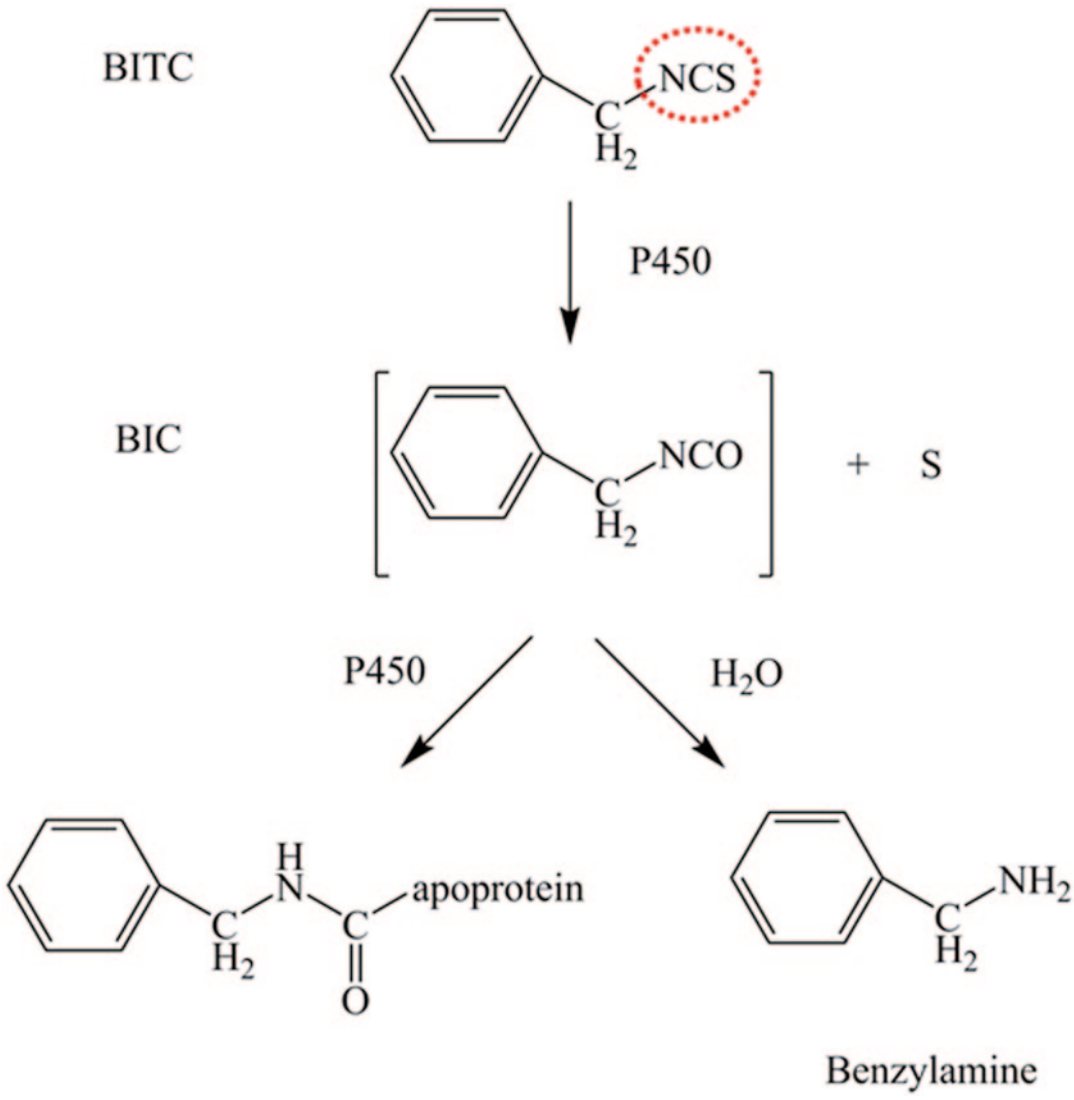


Fig. 5.14 CYP2B1-inactivation by BITC. The pathway proposed for the metabolism of BITC by CYP2B1 leads to the formation of a protein adduct. The circled area indicates the site of metabolism

not only to be potent inhibitors of CYP2A6 and CYP2A13, but to also be mechanismbased inactivators through the formation of adducts with the apoprotein [360]. For both CYP2A6 and CYP2A13 the inactivations showed NADPH-, time-, and concentration-dependence, suggesting that the inactivations were mechanism-based. Since CYP2A6 and CYP2A13 are thought to play an important role in the activation of some tobacco specific chemical carcinogens such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, it was suggested that the isocyanates might be developed as chemopreventive agents to protect those smokers who are unwilling or unable to quit smoking against lung cancer.

BITC also was a potent mechanism-based inactivator of P450s 2B1 from rat and 2E1, from rabbit in the reconstituted systems [361–363]. The losses in activity were time-, concentration-, and NADPH-dependent. Kinetic constants describing the inactivation of CYP2B1 by BITC were K_I, 5.8 μM, k_{inact}, 0.66 min^{-1} and for

CYP2E1 they were K_I, 13 μM, k_{inact}, 0.09 min^{-1}. The inactivation was due to the binding of a reactive intermediate of BITC to the CYP2B1 and CYP2E1 apoproteins. Although a loss in the P450 CO-spectrum was observed, there was no loss in the absolute spectrum from 350 to 600 nm following inactivation of CYP2B1. For CYP2E1, although a loss in the reduced CO-spectrum was observed, there was essentially no loss in the absolute spectrum of the modified protein or in the heme peak detected by HPLC analysis at 405 nm. Nucleophilic scavengers such as GSH, DTT, or potassium cyanide (KCN) were included in the inactivation mixture in attempts to determine if reactive intermediates were escaping the CYP2B1 active site and binding elsewhere on the P450 apoprotein, or possibly to the CPR, resulting in a loss of activity due to the binding of a reactive intermediate at sites other than the active site [361]. The addition of GSH (10 mM) during the inactivation reaction completely abolished the ability of BITC to inactivate CYP2B1. However, this appeared to be due to the rapid formation of a thiocarbamate between the BITC and GSH. This product could be spectrally detected by its UV absorbance at 270 nm [361]. The possibility that the reactive intermediate of BITC inactivates the CPR rather than the P450 could be ruled out, since inclusion of additional CPR in the inactivated system after the removal of residual BITC by dialysis did not restore any enzymatic activity. HPLC analysis of samples incubated with [^{3}H] BITC demonstrated that labeling of the protein in the presence of NADPH increased only in the P450 containing fraction. For CYP2B1 the stoichiometry of BITC binding to P450 was approximately 0.9:1 [361]. Identification of the metabolites of BITC generated by CYP2B1 showed that benzylamine accounted for approximately 50% of the total metabolites formed, with lesser amounts of benzoic acid, benzaldehyde, *N, N′*-dibenzylurea, and *N, N′*-di-benzylthiourea [364]. Therefore, the reactive moiety responsible for the inactivation of CYP2B1 appears to be the benzyl isocyanate intermediate (Fig. 5.14). The BITC-inactivated CYP2E1 exhibited a mass increase of 155 Da, suggesting that the reactive intermediate of BITC responsible for forming an adduct with the apoprotein and inactivating the P450 was either the entire BITC molecule, possibly linked by a disulfide bridge to the apoprotein, or a hydroxylated form of BIC. Incubations of the inactivated CYP2E1 with β-mercaptoethanol did not decrease the amount of radiolabeled BITC bound to CYP2E1, indicating that the protein adduct was not a disulfide-linked BITC molecule [363].

Although the amino acid residue modified by the BITC reactive intermediate has not yet been identified, interesting results were obtained when a CYP2E1 mutant wherein the conserved Thr303 residue was replaced by Ala, was incubated with BITC, PEITC, and *tert*-butylisothiocyanate (tBITC) [363]. Whereas wild-type CYP2E1 was inactivated by all three isothiocyanates, the Thr303 mutant was only inactivated by PEITC and tBITC [363]. This observation was of great interest since the only difference between PEITC and BITC is an additional methylene group in PEITC. Surprisingly, LC–MS analysis suggested the covalent binding of a reactive intermediate of BITC to the CYP2E1 mutant with the mass of 165 Da as compared to a mass of 154 Da for the wild-type enzyme adduct. This mass difference could be due to the addition of a BIC adduct (134 Da) together with a sulfur (32 Da) adduct. Alternatively, the addition of 166 Da to the CYP2E1 apoprotein could result from an adduct consisting of the entire hydroxylated BITC molecule (363).

Thr303 has been shown to be highly conserved in P450s and is generally thought to play a role in catalysis, possibly by serving as a proton donor, and also in substrate interactions. Replacing the Thr303 residue of CYP2E1 did not abolish the 7-ethoxycoumarin (7-EFC) or *p*-nitrophenol activity of the enzyme. Possibly there is enough flexibility in the active site to allow Thr304 to act as a substitute for Thr303. Alternatively, replacing the Thr by Ala may lead to an alteration in the preferred orientation of BITC in the active site, so that the inactivating BIC product is not formed. Since Ala lacks the hydroxyl group of the Thr, it would prohibit the covalent binding of a reactive intermediate to the Ala303 site. On the other hand, when the Thr303 is present, formation of an adduct with the reactive intermediate of BITC

may interfere with the postulated function of that threonine in the proton relay or with other critical architectural arrangements at the active site, such as the formation of hydrogen bond networks. Comparison of the structures of BITC, BEITC, and tBITC reveals that when the bulky positions of the molecules are aligned, the isothiocyanate moiety is aligned very differently in PEITC and tBITC when compared to BITC [363].

tBITC was a more specific mechanism-based inactivator of CYP2E1 than of CYPs 1A1, 1A2, 3A2 or members of the CYP2B family [364, 365]. For CYP2E1 in the purified, reconstituted system, the K_I was 7.6 μM, the k_{inact} was 0.7 min^{-1}, and the $t_{½}$ was 2.6 min [365]. The Thr303Ala mutant exhibited similar values. The inclusion of b_5 in the reconstituted system caused an alteration in the kinetic constants so that they approximated those seen with microsomes (with b_5: K_I=14 μM,k_{inact}=0.38 min^{-1}, and the $t_{1/2}$=1.9 min; in microsomes: K_I=11 μM,k_{inact}= 0.72 min^{-1}, $t_{1/2}$=1.0 min). Although GSH addition to the BITC-inactivation mixture prevented CYP2E1 inactivation, the addition of GSH to the tBITC-inactivation mixture only slowed the rate of reaction suggesting that the reactivity of the two compounds differs in the direct formation of a thiocarbamate with GSH. In addition, the inactivation of CYP2E1 by tBITC showed a direct correlation between the percent loss in the ability of the tBITC inactivated CYP2E1 to form a reduced CO complex and the loss in percent activity. However, no loss was observed in the absolute spectrum, the amount of heme recovered by HPLC analysis at 405 nm, or in the pyridine hemochrome content. Similar results have been observed for the inactivation of CYP2E1 by 3-amino-1,2,4-triazole [366]. The loss in the ability of the tBITC-inactivated CYP2E1 to form the CO complex could be reversed by incubation of the inactivated protein with dithionite for up to 1 h [367]. In addition to restoring a significant amount of the ability to form the reduced CO complex, the inactivated enzyme also regained catalytic activity to the same extent after treatment with dithionite. It has previously been reported [368] that prolonged incubation of dithionite with P450s leads to heme destruction due to the generation of hydrogen peroxide as well as other radicals. Therefore, it appeared that tBITC may block a site on the enzyme responsible for this process, thereby protecting the inactivated CYP2E1 from heme destruction. The removal of the tBITC blocking moiety with the restoration of the ability to form the reduced CO complex once again made the P450 susceptible to dithionite bleaching of the heme [367]. Mechanistically, the inactivation of CYP2E1 by tBITC was not due to the inability of the enzyme to be reduced initially or because either of the two CPR-dependent steps were impaired [367]. However, the inactivation did result in a decreased ability of the CYP2E1 to bind the substrate/inhibitor 4-methylpyrazole. Spectral analysis of the inactivated sample by EPR demonstrated that it consisted of at least two populations [367]. Approximately 24 % of the inactivated CYP2E1 was EPR silent indicating that this population of the enzyme was in the Fe^{2+} state, suggesting it had been trapped in this state, thereby preventing it from completing the normal catalytic cycle. Forty-four percent of the remaining fraction gave an unusual low spin EPR signal which is believed to be due to displacement of a water molecule from the sixth ligand of the heme by an adduct formed with the reactive intermediate of the tBITC. Analysis of the tBITC-inactivated CYP2E1 using LC/MS showed an increase in mass of 118 Da from 53,804 ± 2 Da for the native enzyme to 53,922 ± 2 Da for the inactivated CYP2E1. This mass increase is consistent with the formation of an adduct between the entire tBITC molecule and the CYP2E1 apoprotein via a disulfide linkage with one of the four cysteines in CYP2E1. Presumably, this disulfide-linked tBITC molecule is removed by prolonged incubation with dithionite. Involvement of Cys378, which forms the fifth ligand to the heme iron, and Cys488 at the C-terminus can most likely be ruled out, leaving Cys174 and Cys261 as the remaining possible candidates for protein modification by tBITC. These data suggest that tBITC binds to a critical amino acid residue in the active site and this amino acid residue is presumably in the vicinity of the sixth axial ligand binding site to the heme and thereby interferes with oxygen

binding, substrate binding, and the binding of CO to the reduced protein [367].

PEITC, a naturally occurring isothiocyanate which has been shown to be a potent cancer chemopreventative agent, is a mechanism-based inactivator of human CYP2E1 [369]. The inactivation was shown to be concentration-, NADPH-, and time-dependent. The K_I, k_{inact}, and $t_{1/2}$ values for the inactivation of the 7-EFC catalytic activity were determined to be 11 μM, 0.23 min^{-1}, and 3.0 min, respectively. Cytochrome b_5 had no effect on the K_I or k_{inact} for the reaction. The partition ratio was 12, the inactivation was not inhibited in the presence of GSH, and there was no reversal of inactivation by dialysis. CYP2E1 inactivation by PEITC is due to both destruction of the heme prosthetic group and protein modification, with the latter being the primary pathway for the inactivation. GSH-adducts of phenethylisocyanate (PIC) and phenethylamine were observed during the metabolism by CYP2E1, indicating that PIC is formed as a reactive intermediate following the P450-catalyzed desulfurization of PEITC. Incubation of CYP2E1 with PIC in the absence of NADPH showed covalent binding resulting in the formation of protein adducts, but there was no inactivation of the P450. Electrospray ionization–liquid chromatographic mass spectrometric (ESI–LC–MS) analysis of the inactivated CYP2E1 suggested that the inactivation of CYP2E1 is due to reaction with a reactive sulfur atom generated during PEITC desulfurization. The mass increase of the apoprotein of 147 Da after incubation with PIC is the result of the formation of a covalent adduct in the absence of metabolism. Following incubation of CYP2E1 with PEITC in the reconstituted mixture, the PEITC-inactivated CYP2E1 showed a mass increase of 175 ± 6 mass units, which is larger than that for the PIC-derived apoprotein adduct with a mass difference of 147 Da, and is consistent with the mass of a PIC-derived protein adduct plus one sulfur atom (147 + 32 Da). Alternatively, this mass difference could also be accounted for by reaction with an intermediate that resulted from the formation of a covalent adduct with the oxidized PEITC (PEITC, 163 Da plus one oxygen atom, 16 Da). Trypsin digestion of the inactivated CYP2E1 followed by LC–MS/MS resulted in the identification of a peptide with the sequence DLTDCLLVEMEK, corresponding to residues 264–275 of human CYP2E1 and residue Cys268 was shown to be the residue modified by PIC [369].

Alkyl xanthates are derivatives of dithicarbonic acid ($ROCSS^-K^+$). A number of xanthates have been shown to be specific mechanism-based inactivators of P450 enzymes both in microsomal systems and in reconstituted systems [370–372]. Studies on the effects of a number of xanthates on the enzymatic activities of CYPs 1A1, 2B, 2C9, 2D6, 2E1, 3A2, and 3A4 have been examined. Several of the xanthates were shown to be particularly effective mechanism-based inactivators of CYPs 2B1 and 2B6. The inactivation kinetics showed a dependence on the length of the alkyl chain link (C2–C20). With the exception of isopropyl xanthate, the general trend was that with increasing chain length, the inactivation rates slowed down. CYP2E1 was also inactivated by xanthates but at concentrations that in general were 2–3 fold higher than those required for inactivation of the members of the CYP2B family. *N*-octylxanthate (C8) appeared to be the most potent inactivator of both CYPs 2B1 and 2B6. *n*-Propylxanthate (nPX) inactivated the 7-EFC activity of CYP2B1 or CYP2B6 in a mechanism-based manner. The inactivations were concentration-, NADPH-, and time-dependent. The K_I for CYP2B1 was 44 μM and the k_{inact} was 0.2 min^{-1}. For CYP2B6, the K_I was 12 μM and the k_{inact} was 0.6 min^{-1}. Incubation of CYP2B1 with nPX and NADPH for 20 min resulted in a 75% inactivation of the enzyme with a concurrent 25% loss in the ability to form the reduced CO complex, even though there was very little loss in the absolute spectrum of the inactivated CYP2B1. With CYP2B6, there was an 83% loss in enzymatic activity with only a 12% loss in the CO-reduced spectrum. The partition ratio for nPX inactivation of CYP2B1 was 32. The stoichiometry for labeling of the CYP2B1 by radiolabeled nPX was 1.2:1. Significant enzyme activity could be restored to the nPX-inactivated CYP2B1 when iodosobenzene was used as the alternative oxidant in place of NADPH and O_2. These results suggest that the adduct formed by the nPX reactive intermediate was with an amino acid residue critical for a CPR-dependent step. Alternatively,

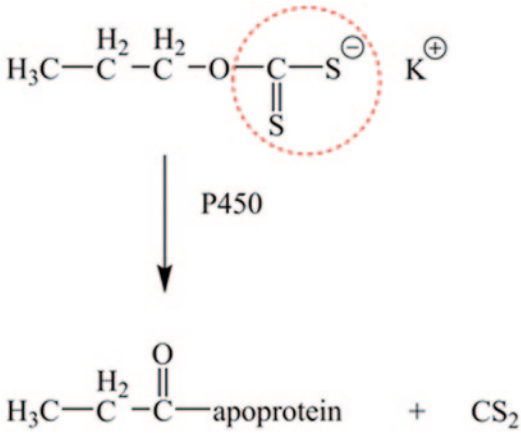


Fig. 5.15 The pathway proposed for the metabolism of *N*-propylxanthate by CYP2B1 leading to the formation of a reactive intermediate, which then forms a protein adduct. The circled area indicates the initial site of metabolism

it is possible that the modification of the amino acid residue by nPX may have disrupted a proton transfer step required to generate the oxy-ferryl intermediate. A third possibility is that the modification may have altered either the binding or the dissociation of the substrate and in some way favored oxidation supported by iodosobenzene. Although the reactive intermediate of nPX responsible for the inactivation of CYPs 2B1 and 2B6 has not yet been identified, it has been suggested that the initial oxidation by CYP2B1 is on the α-carbon of nPX and that the inactivating species could be a hydroxylated propyl radical or propylketene (Fig. 5.15) [370].

Fifteen xanthates with carbon chains of various lengths or having different substitutions were assessed for their ability to inactivate CYPs 2B1 and 2B6. All 15 of the xanthates were found to be mechanism-based inactivators of CYPs 2B1 and 2B6 [371]. All of them inactivated CYP2B1 in a time- and concentration-dependent manner and the rates of inactivation ranged from 0.02 to 0.22 min^{-1}. The concentrations required for half-maximal rates of inactivation ranged from 2.4 to 69 μM. The general trend in the inactivation reactions suggested that longer carbon chains led to slower rates of inactivation with longer half times of inactivation and higher partition ratios. For CYP2B1 the most effective inactivators were those with intermediate length substitutions. The best inactivator of CYP2B1 was the C8 xanthate having a K_I of 2.4 μM, a k_{inact} of 0.07 min^{-1}, and a partition ratio of 4 [371]. Four of the xanthates were examined further for their ability to serve as mechanism-based inactivators of CYP2B6 [371]. Once again, the C8 xanthate was the most effective inactivator with a K_I of 1 μM. Although the K_I values were generally lower than those for CYP2B1, the k_{inact} values were generally three- to fivefold slower. CYP2E1 was inactivated by the xanthates at concentrations that were 15- to 100-fold higher than those required for CYPs 2B. None of the xanthates tested were able to act as mechanism-based inactivators of CYP1A1, CYP2C9, CYP2D6, CYP3A2, or CYP3A4.

The mechanism by which alkyl xanthates inactivate CYP2B1 was investigated by examining the effects of C8 on the individual steps of the CYP2B1 catalytic cycle [372]. Dramatic losses in the 7-EFC activity of CYP2B1 were observed when it was incubated with five different xanthates in the presence of NADPH. With the exception of the C14 xanthate, there was virtually no loss in the heme absorbance at 418 nm or in the absorbance of the reduced-CO complex at 450 nm. The long-chain xanthates reduced the rate of the transfer of the first electron in the P450 catalytic cycle by stabilizing the heme in its low spin state. C8 led to very little formation of the oxy-ferryl intermediate complex. The rates of reduction of the native, C8-exposed, and C8-inactivated CYP2B1 by CPR were measured [372]. The rate of reduction of the C8-inactivated P450 was approximately 62 % slower when compared to that of the native enzyme either in the absence or presence of benzphetamine. The formation of products from benzphetamine by the three enzyme preparations was determined [372]. The C8-inactivated CYP2B1 exhibited a much lower rate of NADPH consumption and formation of the formaldehyde product. In addition, the ratio of H_2O_2 to formaldehyde increased from 1:1 for the unmodified enzyme to 2.8:1 for the inactivated CYP2B1 [372]. Thus, these observations suggest that the reactive intermediate formed from the C8-xanthate causes covalent modification of the CYP2B1 apoprotein, which reduces

the rate of the first electron transfer by CPR and also leads to the uncoupling of product formation from electron transfer by diverting a greater proportion of the electrons to the formation of H_2O_2 rather than product formation [372].

Disulfiram (Antabuse) has been used therapeutically for the treatment of alcoholism for more than 60 years because of its ability to inhibit aldehyde dehydrogenase. Another enzyme that is inhibited by disulfiram is human CYP2E1 [373]. The inhibition of CYP2E1 by disulfiram has previously been reported to be due to MBI by a reactive intermediate formed by CYP2E1 which reacts with the enzyme protein. Recently, it has been demonstrated that disulfiram by itself does not inactivate CYP2E1 in an in vitro reaction; however, a metabolite of disulfiram, diethyldithiocarbamate (DDC) is converted to a reactive intermediate by CYP2E1 and that intermediate subsequently inactivates the protein leading to MBI [373]. LC–MS of the inactivated CYP2E1 demonstrates that the inactivation results from the formation of an adduct of the reactive metabolite of DDC with the apoprotein. MS studies of the GSH-adduct formed by the reactive intermediate indicate that the reactive intermediate has a mass of 116 Da. HPLC analysis of the inactivated protein mixture showed no change in the amount of unmodified heme or the presence of any modified heme [373]. These results suggest that binding of the reactive intermediate to the apoprotein involves formation of a disulfide bond with one of the eight cysteines in CYP2E1. Incubation of the modified protein in the presence of DTT resulted in the loss of the DDC adduct and reversal of the mass of the CYP2E1 to that of the unmodified protein. However, no regain of activity following loss of the DDC adduct could be observed. These results support the hypothesis that adduct formation leads to a disulfide bond. In addition to investigating the inactivation of wild-type CYP2E1, the inactivation of two of its polymorphic mutants, CYP2E1.2 and CYP2E1.4 was also investigated. For the wild-type enzyme, the K_I was 12.2 μM and the k_{inact} was 0.02 min^{-1}. The K_I values for the two polymorphic mutants were 227.6 and 12.4 μM for CYP2E1.2 and CYP2E1.4 and the k_{inact} values were 0.0061 and 0.0187 min^{-1}, respectively. These results demonstrate that DDC is much less efficient as an inactivator of CYP2E1.2 than it is of either the wild-type or the CYP2E1.4 variant [373].

Ticlopidine (Fig. 5.11) is a substituted thiophene that has been used clinically as an antiplatelet aggregation agent and has been identified as a mechanism-based inactivator of CYP2C19 [374]. The inactivation is thought to occur as a consequence of *S*-oxidation of the thiophene moiety. The inactivation exhibits the following kinetic parameters: $K_I = 97$ μM, $k_{inact} = 3.2 \times 10^{-3}$ s^{-1}, and the partition ratio is 126. Studies with recombinant human P450s in Supersomes™ indicate that CYP2B6 is even more effectively inactivated than CYP2C19, not only by ticlopidine, but also by clopidogrel, a related thienopyridine antiplatelet aggregating agent [374]. The inactivation of CYP2B6 was time-, concentration-, and NADPH-dependent and it was irreversible upon dialysis [374]. For clopidogrel the K_I and k_{inact} for CYP2B6 were 1.1 μM and 1.5 min^{-1}, and for ticlopidine the K_I was 4.8 μM and the k_{inact} was 0.8 min^{-1} [374]. The inactivations were inhibited by the presence of alternative substrates but not by scavengers of reactive oxygen or trapping agents for reactive electrophiles.

The antiplatelet activity of clopidogrel requires metabolic biotransformation to a pharmacologically active metabolite by P450s [375]. The active metabolite contains a reactive thiol group that covalently modifies the Cys97 and Cys175 residues of the human $P2Y_{12}$ ADP receptor via the formation of disulfide bonds to prevent the adenosine diphosphate (ADP)-induced platelet aggregation [376]. The bioactivation of clopidogrel to the active metabolite is believed to occur in two sequential oxidative steps. The first oxidative step involves insertion of a single oxygen atom into clopidogrel to give 2-oxo-clopidogrel, a thiolactone metabolite. The second oxidative step involves further bioactivation of the thiolactone metabolite to produce the active metabolite (Fig. 5.16).

Clopidogrel and its thiolactone metabolite, 2-oxo-clopidogrel, both inactivate human CYP2B6 in a time- and concentration-dependent

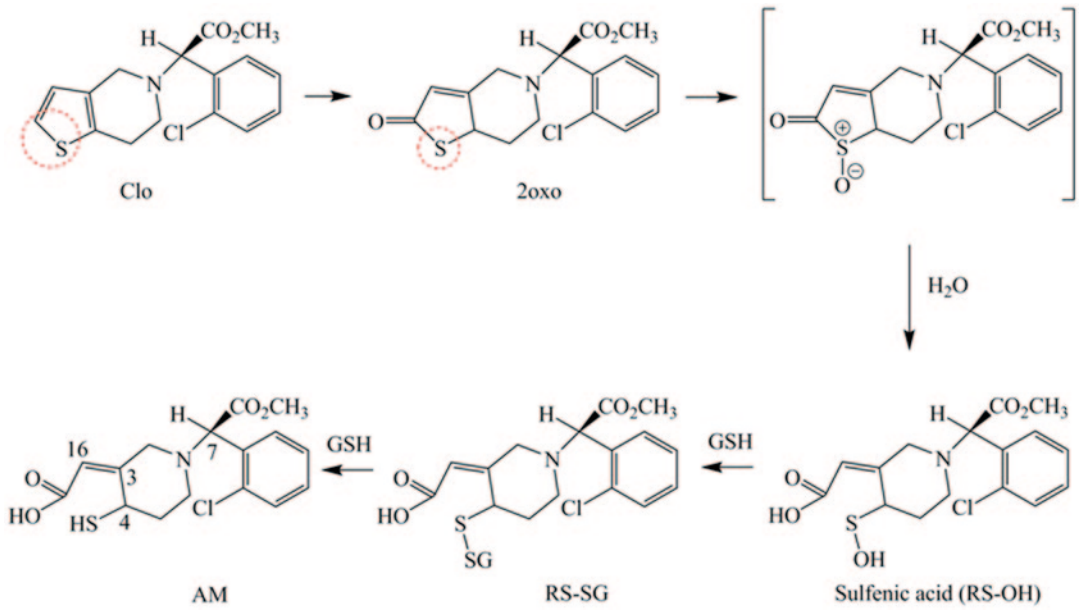


Fig. 5.16 The pathway for the bioactivation of clopidogrel by P450s: Clo, clopidogrel; 2 oxo, 2-oxo-clopidogrel. The numbers shown in the structure of the active metabolite (AM) indicate the numbering of the two chiral centers (C4 and C7) and the exocyclic double bond (C3 and C16). The circled areas indicate the sites of metabolism by the P450s

manner [377]. The K_I and k_{inact} values for clopidogrel were 2.4 μM and 0.17 min^{-1}, respectively, whereas for 2-oxo-clopidogrel, the K_I and k_{inact} values were 6.3 μM and 0.092 min^{-1}, respectively. LC–MS analysis of the CYP2B6 protein inactivated with either clopidogrel or 2-oxo-clopidogrel showed a mass increase of ~350 Da corresponding to the addition of the active metabolite of clopidogrel to the protein [377]. This adduct could be cleaved from the protein by incubation with DTT, confirming that the active metabolite is covalently bound to a cysteine residue via a disulfide bond. Tryptic digestion of the inactivated CYP2B6 followed by ESI–LC–MS/MS of peptides derived from tryptic digestion identified Cys475 as the site of covalent modification by the active metabolite [377]. This was confirmed by studies in which Cys475 was mutated to a serine residue, which eliminated the MBI of the mutant by 2-oxo-clopidogrel and also prevented formation of the protein adduct. However, this mutation did not prevent the mutant from being inactivated by clopidogrel. Interestingly, the inactivation of both the wild-type CYP2B6 and the mutant by clopidogrel, but not by the 2-oxo-clopidogrel, led to the loss of heme, which accounts for most of the loss of the catalytic activity. Therefore, it was suggested that clopidogrel inactivates CYP2B6 primarily through destruction of the heme whereas 2-oxo-clopidogrel inactivates through covalent modification of Cys475 [377].

Studies on the metabolism of clopidogrel by human liver microsomes in the presence of four reductants: GSH, *L*-cysteine, *N*-acetyl-*L*-cysteine (NAC), and ascorbic acid demonstrated that formation of the active metabolite was greatly affected by the reductant used [378]. In the case of GSH, the formation of the active metabolite and the glutathionyl conjugate was dependent on the GSH concentration, which indicates that formation of the thiol conjugates constitutes an integral part of the bioactivation processes for clopidogrel. The active metabolite was slowly converted to the thiol conjugate with a half-life of ~10 h. Addition of DTT to the reaction mixture reversed the conversion, resulting in a decrease in the active metabolite-thiol conjugate levels and a concomitant increase in the levels of the active me-

tabolite. These results confirm that the active metabolite was formed through oxidative opening of the thiol lactone ring and suggest the existence of an equilibrium between the active metabolite, the thiol conjugates, and the reductants [378].

One of the first chlorinated mechanism-based inactivators demonstrated to act by irreversibly modifying the protein was chloramphenicol [299–302]. Binding of the [^{14}C]-labeled chloramphenicol to the apoprotein correlated with the loss of the CYP2B1-dependent 7-EFC activity, and proteolytic digestion of the inactivated CYP2B1 yielded a single [^{14}C]-modified amino acid residue [299–302]. Hydrolysis of the modified amino acid residue yielded lysine and a fragment of the chloramphenicol indicating that chloramphenicol was converted to an oxamyl chloride intermediate that then could either modify a critical lysine residue on the protein or be hydrolyzed to give the oxamic acid. Acylation of the lysine residue is suggested to inhibit the transfer of electrons from CPR to CYP2B1, since the inactivated enzyme was still catalytically active in the presence of either iodosobenzene or cumene hydroperoxide [302]. The observation that the 7-EFC activity is not inhibited at all by the presence of chloramphenicol when activated oxygen donors are used suggests that the chloramphenicol is not covalently bound in the substrate-binding site.

The selectivity of chloramphenicol and several of its analogs in the inactivation of various P450 isozymes has been reported [379]. Chloramphenicol was found to inactivate rat liver microsomal CYP2B1>CYP3A >CYP2C11 >CYP2A1 as assayed with androstenedione hydroxylation as the functional probe. The selectivity of the chloramphenicol analogs for MBI of P450 was determined by at least three structural features: (a) substitutions on the ethyl side chain; (b) the presence of a *para*-nitro group on the phenol ring; and (c) the number of halogen atoms. Thus, while *N*-(2-*p*-nitrophenethyl)- and *N*-(1,2-diphenethyl)-dichloroacetamide both inactivated CYP3A4 readily, the analog *N*-(2-phenethyl) dichloroacetamide did not inactivate CYP3A even though it was a reversible inhibitor [379]. The addition of a second phenol at the 1- or 2-position of the phenethyl side chain or of a *para*-nitro or -bromo substituent on the phenol ring gave compounds that were selective inactivators of CYP2B1 over CYP2C11, CYP2C6, or CYP2A1 [304]. Therefore, *N*-(2-*p*-nitrophenethyl)-dichloroacetamide and *N*-(2-*p*-bromophenethyl)-dichloroacetamide were the two most effective and selective inactivators of CYP2B1 both in vitro and in vivo [304].

21-Chloropregnenolone, 21,21-dichloropregnenolone, and 21,21-dichloroprogesterone have all been shown to be mechanism-based inactivators of various P450s [380, 381]. The 21,21-dichloropregnenolone and the 21,21-dichloroprogesterone showed very similar k_{inact} values of approximately 0.1 min^{-1} for the inactivation of rat liver microsomal CYP3A enzymes when measured with both progesterone or androstenedione as the probe substrates. The 21,21-dichloroprogesterone was even more efficient at inactivating CYP2C6 with a k_{inact} of ~0.2 min^{-1}. The 21,21-dichloropregnenolone was also a good mechanism-based inactivator of rabbit liver CYP2C5, but not of rabbit adrenal CYP21 [381]. However, CYP21 was rapidly inactivated by 21,21-dichloroprogesterone, indicating that the replacement of a methyl group that may normally be oxidized by a P450 by a dichloromethyl functional group may prove to be of value in designing specific inhibitors for specific P450s.

5.3.3.2 Olefins and Acetylenes

A variety of compounds containing an olefinic bond, such as ethylene, allylisopropylacetamide (AIA), and secobarbital, can form covalent adducts on the nitrogen of the porphyrin group of the prosthetic heme leading to inactivation [382–385]. Secobarbital has been shown to completely inactivate CYP2B1 with only partial loss of the heme chromophore [384, 386, 387]. Isolation of the modified CYP2B1 protein and the *N*-alkylated porphyrins indicates that the reactive compound partitions between protein modification, *N*-alkylation of the heme, and formation of an epoxide metabolite in the ratio of 0.2:0.8:59, respectively [387] (Fig. 5.17). The formation of a heme adduct in the active site of CYP2B1 was confirmed spectrally based on its typical absorption maximum at ~445 nm, a characteristic fea-

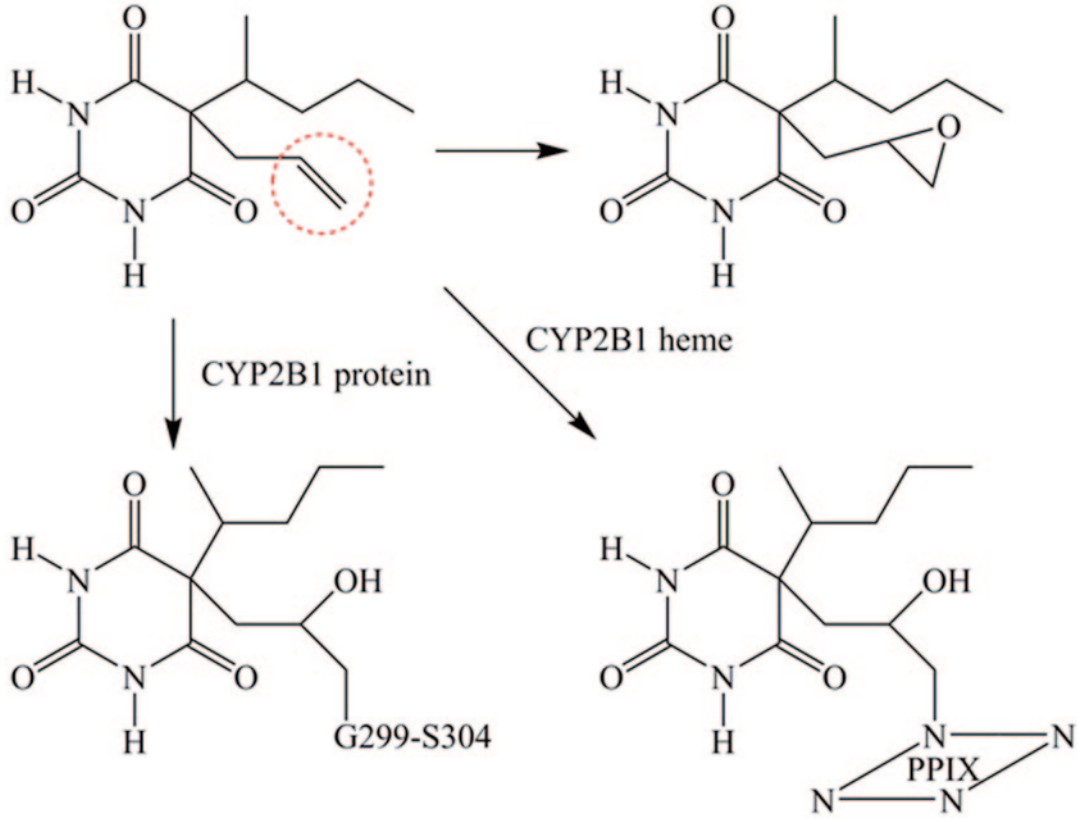


Fig. 5.17 Pathways for the oxidation of secobarbital by CYP2B1 leading to alkylation of the protein on the peptide spanning residues Gly299 to Ser304 and *N*-alkylation of the heme group, as well as the generation of an epoxide. The circled area indicates the initial site of metabolism by CYP2B1

ture of iron complexed *N*-modified porphyrins [388]. The modified CYP2B1 peptide has been isolated and shown to span residues 277–323. By sequence analogy, these residues correspond to the distal I helix in $P450_{cam}$ [386, 389–392]. Further digestion of the modified peptide has resulted in identification of the site for modification by secobarbital to a residue in the peptide G299-S304 [387]. Although the identity of the adducted residue has not yet been determined, these results are consistent with modification of the CYP2B1 in the active site. Specific mutations of CYP2B1 in the putative substrate-recognition site (SRS) 2,4,5, and 6, but not in SRS-1, cause a decrease in the inactivation by secobarbital. Mutation of residue 367 from V to A in SRS-5 had a marked inhibitory effect on protein modification [387]. Isolation of the *N*-modified porphyrins as the parent adducts as well as the corresponding dimethylesters and analysis by LC–MS demonstrated the formation of adducts of hydroxysecobarbital with protoporphyrin IX (Fig. 5.17) [386].

Like terminal olefins, terminal acetylenes can alkylate the P450 prosthetic heme. However, compounds such as 10-undecynoic acid, 1-ethynylpyrene, 2-ethynylnaphthalene, 9-ethynylnaphthalene, 17β-ethynylprogesterone, and 17α-ethynylestradiol (EE) inactivate P450s primarily by covalently binding to the apoprotein with little or no effect on the heme group (Fig. 5.18) [305, 310, 317, 318]. Almost stoichiometric binding of 10-undecynoic acid to rat liver CYP4A1 (the ω-hydroxylase) as well as of 2-ethynylnaphthalene and 1-ethynylpyrene to CYP1A1 and -1A2, and of EE to CYP3A4 has been observed [305–310, 314–318]. Isolation of

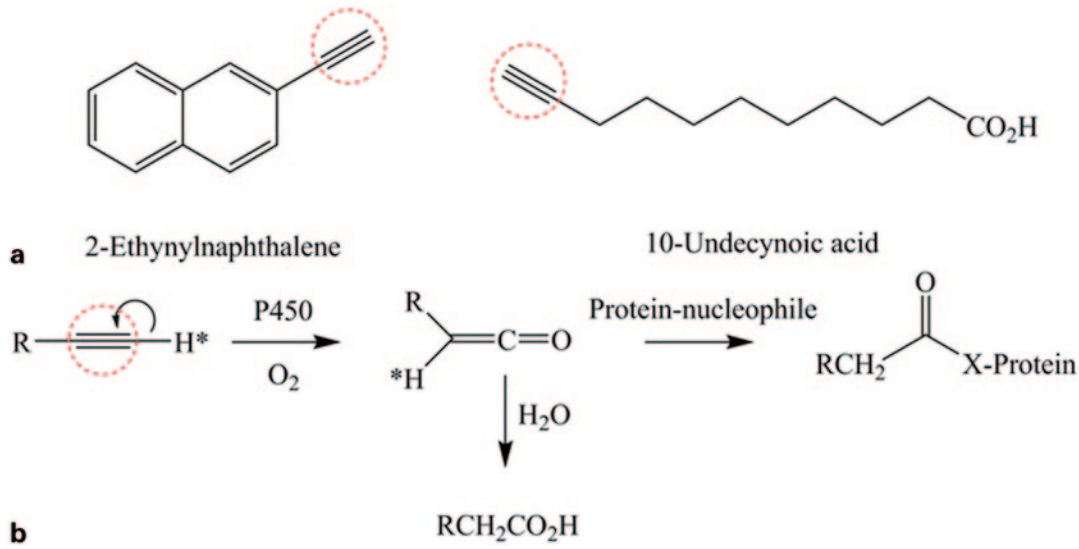


Fig. 5.18 Metabolic inactivation by 2-ethynylnaphthalene and 10-undecynoic acid. **a** Structures of both compounds, which are known to inactivate P450 enzymes, presumably through the formation of a ketene intermediate as shown in **b**. **b** The oxidation of terminal acetylenes is thought to lead to the formation of ketene intermediates, which then can react with active-site nucleophilic residues, inactivating the P450 involved. They can also react with water to give carboxylic acids, as shown. The circled areas indicate the sites of metabolism leading to the ketenes

acidic metabolites from incubations of 10-undecynoic acid (Fig. 5.18) and 1-ethynylpyrene provides strong support for the formation of a reactive intermediate following oxygen transfer from the P450 heme to the terminal carbon of the triple bond, which then triggers migration of the terminal hydrogen to the adjacent carbon (Fig. 5.18). Migration of this hydrogen results in the generation of a reactive ketene which can either acylate the protein or be hydrolyzed to give the carboxylic acid metabolite [310]. The intermediacy of ketenes in the MBI of various P450s has also been suggested for the acylation of bovine adrenal CYP21 by 17β-ethynylprogesterone [312, 313] and of CYPs 1A2, 2B1, and 2B4 by 2-ethynylnaphthalene (Fig. 5.18) [307, 309, 319]. 2-Ethynylnaphthalene inactivates CYP2B1 with a K_I of ~0.08 μM, k_{inact} of 0.83 min^{-1} and a partition ratio of ~4–5 mol of acid formed per inactivation of the CYP2B1 [309].

Addition of the activated oxygen to the internal carbon of the triple bond rather than the terminal carbon of phenylacetylene results in heme *N*-alkylation of CYP2B1 rather than protein acylation [310, 393]. The observation that the phenylacetylene inactivates CYP2B1 primarily via heme alkylation [393] whereas 2-ethynylnaphthalene inactivates primarily by acylation of the protein [307, 309, 319] suggests that the fit of the inhibitor within the active site may be a significant determinant of the particular inactivation mechanism. Although both of these aryl acetylenes yield ketene metabolites, only that formed from the 2-ethynylnaphthalene is able to form a covalent adduct with the CYP2B1 protein [309, 319]. Acylation of this protein by 2-ethynylnaphthalene demonstrates that the inability of phenylacetylene to acylate the apoprotein is not due to the lack of appropriate nucleophilic residues in the active site. Furthermore, confirmation that a ketene is formed as an intermediate during the reaction comes from the observation that phenylacetic acid is formed as a product of the phenylacetylene [309, 319]. The inactivation of CYP2B1 by modification of the protein by the 2-ethynylnaphthalene and heme modification by the phenylacetylene suggest that: (a) the binding of the 2-ethynylnaphthalene in the P450 ac-

tive site is in such an orientation that it prevents delivery of the activated oxygen to the internal carbon and (b) alkylation of the heme by the phenylacetylene is sufficiently efficient relative to the acylation of the apoprotein by the phenyl ketene metabolite that the enzyme is unable to carry out further metabolism before the acylation of the protein becomes significant. These differences presumably are due to the fact that the two agents bind in differential orientations within the CYP2B1 active site or they may have very different binding affinities.

Incubation of radiolabeled 2-ethynylnaphthalene with rat and rabbit CYPs 1A2 followed by tryptic digestion, peptide mapping, and amino acid sequence analysis of the labeled peptides indicated that the inactivation was due to adduct formation on a peptide spanning residues 67–78 in the rat protein and 175–184 in the rabbit protein [307]. However, identification of the actual residue that was modified in each case and the nature of the covalent linkage to the inhibitor could not be determined due to the instability of the P450 peptide adducts [307]. The fact that 2-ethynylnaphthalene modified two very different peptides in the P450s having very similar primary sequences and that it did not inactivate the highly related human CYP1A2 is of interest. Based on alignments of the labeled peptides with the sequence of $P450_{cam}$ (CYP101) the labeled peptide regions 67–78 and 175–184 were suggested to correspond to the A and D helixes, respectively (See Chap. 1). Therefore, the labeled peptide from rat CYP1A2 may include residues from the substrate-binding regions [389–392].

2-Ethynylnaphthalene has also been shown to be a mechanism-based inactivator of CYP2B1 and CYP2B4 [309, 319]. HPLC analysis revealed that the radiolabeled 2-ethynylnaphthalene was covalently bound to the apoprotein with a stoichiometry of approximately 1.3 mol of 2-ethynylnaphthalene per mol of CYP2B1 inactivated. Amino acid sequencing of the radiolabeled CYP2B1 peptides following cleavage of the protein by cyanogen bromide (CNBr) led to the identification of a radiolabeled peptide that includes residues 290–314 of the protein. An analogous peptide spanning residues 273–314 was obtained with CYP2B4. Both of the modified peptides correspond in sequence to the highly conserved I helix of $P450_{cam}$ (CYP101) that appears to play an important role in forming the active site and contacts both the substrate and the heme group [389–392]. These peptides also contain the highly conserved Thr302. The functional role of Thr302 in the inactivation of CYP2B4 by 2-ethynylnaphthalene was confirmed when it was shown that the Thr302A variant exhibited a significantly slower rate of inactivation (0.05 ± 0.01 min^{-1}) as compared with the rate of inactivation of the wild-type (0.20 ± 0.05 min^{-1}), suggesting that the Thr302 is the acylated residue in CYP2B4. If the hydroxyl group of the threonine is the protein nucleophile that is modified, the resulting adduct would be an ester [394].

9-Ethynylphenanthrene (9EP) has also been shown to be an effective mechanism-based inactivator of CYP2B1 [395]. CYP2B1 inactivation by 9EP was time-, NADPH-, and concentration-dependent. The activity loss followed pseudo-first-order kinetics, with a K_I of 138 nM and a k_{inact} of 0.5 min^{-1}. HPLC and SDS-PAGE analysis demonstrated that radiolabeled 9EP was irreversibly bound to the protein moiety with a stoichiometry of ~0.8 nmol of 9EP bound per nmol of CYP2B1. CNBr cleavage of the radiolabeled CYP2B1 followed by Tricine SDS-PAGE analysis of the peptides resulted in identification of a radiolabeled peptide having a mass of ~3 kDa. Analysis of the radiolabeled peptide using matrix-assisted laser desorotion/ionization (MALDI)-MS showed two peaks at *m*/*z* 2720.9 and 2939.9. The lower mass peak is the molecular ion (MH^+) for the Ile 290-Met 314 peptide (theoretical 2722.2), while the higher mass peak corresponds to the $MH^+$of the modified peptide (theoretical 2940.5). The mass difference between the labeled and unlabeled peptide of ~219 Da would correspond to the addition of a phenanthrylacetyl group to the peptide. Further digestion with pepsin of the fraction containing the modified and unmodified peptides and reanalysis by MALDI-MS showed that the site of attachment could be assigned to one of the amino acid residues in the peptide Phe297 to Leu307 [395]. It was hypothesized that the attachment was probably an ester linkage to one of

the six Thr or Ser residues in that region. Based on sequence alignments with bacterial CYP101, this region is part of the SRS 4.The possibility of an anhydride formation through Glu was ruled out since it would not be expected to survive the slightly acidic conditions used to purify the peptide by HPLC [395].

Subsequent studies were performed to investigate the mechanism by which covalent binding of the phenanthryl acetyl group to the protein moiety inactivated the protein in order to elucidate the possible role(s) of this region in catalysis [396]. For these studies, the abilities of 9-EP-modified and native CYP2B1 to catalyze some of the individual steps of the P450 catalytic cycle were determined. Although inactivation by 9EP results in a 90–95% loss in the NADPH-supported deethylation of 7-EFC, it has no effect on the metabolism of 7-EFC supported by iodosobenzene or cumene hydroperoxide. No decrease was observed in the ability of the modified CYP2B1 to form the steady-state level of the reduced CO complex either enzymatically with NADPH and CPR or chemically with sodium dithionite. However, the rate of reduction by CPR under anaerobic conditions was only 50% of that of the native protein in the absence of substrate and 35% of that of the native protein in the presence of substrate. The 9EP-modified protein exhibited a slower rate of NADPH oxidation, H_2O_2 formation, and the formation of formaldehyde during metabolism of benzphetamine when compared to the native enzyme. The ratio of H_2O_2 to HCHO was 1.0:1.0 for native enzyme and 1.6:1.0 for the modified protein. The ability of the modified protein to form the steady-state level of the iron-oxygen complex in the presence of cyclohexane was decreased. These results are consistent with the idea that the inactivation via adduct formation between 9EP and one of the residues in the Phe297 to Leu307 peptide impairs the reduction of the CYP2B1 by CPR and also results in the uncoupling of NADPH utilization and oxygen consumption from product formation [396].

9EP has also been shown to be a mechanism-based inactivator of CYP2B4 [397]. The k_{inact} and the partition ratio were 0.25 $min^{-1}$and 0.2, respectively [397]. Interestingly, the inactivation exhibited sigmoidal kinetics with an S_{50} of 4.5 μM and a Hill coefficient of 2.5, indicative of homotropic cooperativity. ESI–LC–MS showed that the inactivated apoprotein exhibited an increased mass of 218 Da. This increase is equivalent to the mass of one molecule of 9EP (202 Da) plus one oxygen atom. The mass of the unmodified apoprotein was not observed in the inactivated sample, indicating that the CYP2B4 was completely labeled by 9EP under the conditions used. Although the 9EP-modified CYP2B4 showed a loss of approximately 50% of the CO-detectable heme, no loss of the native heme was observed when the inactivated protein was analyzed by HPLC. The modified CYP2B4 was purified to homogeneity and its structure was determined by X-ray crystallography [397]. The crystal structure showed the 9EP is covalently attached to the Oγ of Thr302 via an ester bond, consistent with the increase in mass of the protein of 218 Da. The bulky phenanthrenyl ring of the 9EP produced inward rotations of Phe206 and Phe297, resulting in the formation of a compact active site. Thus, the binding of a second molecule of 9EP at the active site was prohibited. However, studies on the fluorescence quenching of 9EP by the unmodified or 9EP-modified CYP2B4 showed that there were at least two 9EP binding sites having distinctly different affinities. The lower affinity site was the catalytic site and the higher affinity site was located on the periphery of the protein. Studies using computer-aided docking and molecular dynamics simulations with one or two ligands bound to the protein showed that the higher affinity site (allosteric) is situated at the entrance of a substrate access channel which is surrounded by the F′ helix, the β1–β2 loop, and the β4 loop [397]. The presence of this ligand at the allosteric site enhances the efficiency of the activation of the 9EP- acetylenic group at the active site and its subsequent covalent binding to the Thr302 [397].

7-Ethynylcoumarin (7-EC; Fig. 5.19) was synthesized as a potential mechanism-based inhibitor of CYP2A6, a preferential coumarin7-hydroxylase [398]. Although it showed a minimum ability to serve as a mechanism-based inactivator of CYP2A6, it was an effective inactivator of CYP2B1 [398]. CYP2B1 inactivation dem-

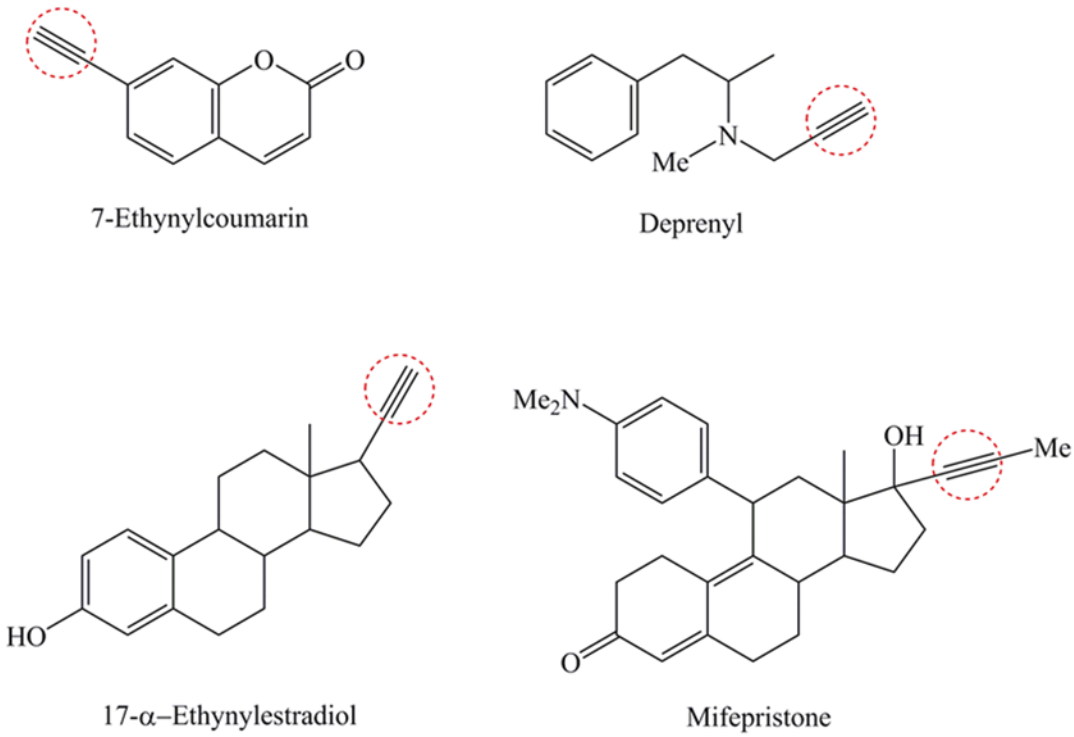
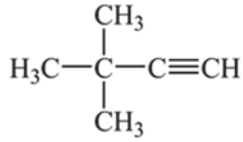


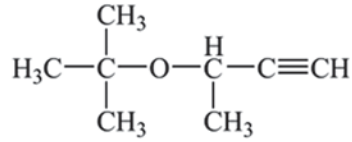
Fig. 5.19 7-Ethynylcoumarin, deprenyl, 17α-ethynylestradiol, and mifepristone. These agents have all been shown to inactivate P450s. It is thought that in all these cases the reactive intermediate arises following oxidation of the triple bond that is circled in the compounds

onstrated pseudo-first-order kinetics and was NADPH- and inhibitor-dependent. The K_I and k_{inact} were 25 μM and 0.39 min^{-1}, respectively, with a partition ratio of 25.Activity loss was not associated with a significant loss in the reduced-CO spectrum, suggesting that the inactivation was primarily due to the modification of the P450 protein rather than the heme. ESI–MS analysis of the inactivated protein demonstrated the attachment of one molecule of the inactivator along with one atom of oxygen in a 1:1 ratio to the apoprotein, which gave a mass difference of 185 Da between the modified and native apo-P450. This is the mass difference that would be expected following the generation of a ketene which then reacts to form an adduct with a nucleophile in the protein. ESI–LC–MS was also used to verify the absence of modified heme as well as the lack of modification of the CPR [398].

Two structurally related compounds containing a *tert*-butyl moiety to increase the specificity for CYP2E1 as well as an ethynyl functional group for metabolic activation by the P450 to a reactive intermediate that could serve as a mechanism-based inactivator, were shown to be mechanism-based inactivators of the CYP2E1 T303A mutant [399]. *tert*-Butyl acetylene (tBA) and *tert*-butyl 1-methyl-2-propynyl ether (tBMP; Fig. 5.20) inactivated the P450s via three different mechanisms: (a) alkylation of the heme prosthetic moiety (inactivation of P450s by tBA and tBMP); (b) a combination of protein and heme alkylation (inactivation of CYP2E1 by tBA); (c) reversible alkylation of the P450 heme which had not previously been described (inactivation of the T303A mutant by tBA).The inactivations were time-, concentration-, and NADPH-dependent [399]. The K_I values for the inactivation of CYP2E1 and the mutant by tBA were 1.0 and 2.0 nM, and the k_{inact} values were 0.20 and 0.38 min^{-1}, respectively. The K_I values for the tBMP-inactivated P450s were 0.1 and 1.0 nM,



tert-butyl acetylene (tBA)



tert-butyl 1-methyl-2-propynylether (tBMP)

Fig. 5.20 Structures of *tert*-butyl acetylene and *tert*-butyl 1-methyl-2-propynyl ether. These two structurally similar acetylenic compounds contain the *tert*-butyl moiety to provide specificity for CYP2B1, as well as an ethynyl functional group for metabolism to give a reactive intermediate that can covalently modify the protein

and the k_{inact} values were 0.12 and 0.07 min^{-1}, respectively. Losses in enzyme activity occurred with concurrent losses in the reduced CO-spectrum and P450 heme and these were accompanied by the appearance of two different tBA- or tBMP-modified heme products. LC–MS analysis of the adducted hemes showed masses of 661 or 705 Da, consistent with the mass of an iron-depleted heme plus the masses of a tBA or tBMP reactive intermediate and one oxygen atom, respectively. However, only the tBA-inactivated wild-type 2E1 exhibited a modified apoprotein having an increase in mass of 99 Da, corresponding to the mass of an adduct of tBA plus one oxygen atom. Surprisingly, the inactivation, loss of the reduced CO-spectrum and P450 heme, and the heme adduct formation of the tBA-inactivated T303A mutant could be completely reversed by dialysis [399]. The characterization of this reversible inactivation mechanism demonstrated that the losses in the native heme and in the catalytic activity of the tBA-inactivated T303A mutant could be restored either by spin column gel filtration or dialysis [400]. The acetylene heme adducts having *m*/*z* values of 661 Da were reversible with time. Interestingly, the retention of stable heme adducts in the tBA-inactivated T303A mutant required a source of exogenous protons, whereas the wild-type CYP2E1 formed stable tBA adducts under the same conditions regardless of prior preacidification [400]. These results suggest an important role for the highly conserved Thr303 residue in donating protons through the CYP2E1 active site and suggest that it may be a possible participant in a proton relay network to the active site and that it plays a role in the stabilization of a reactive intermediate during substrate metabolism by P450s.

Studies with the alternate oxidants *tert*-butyl hydroperoxide (tBHP) and cumene hydroperoxide (CHP) demonstrating that they were capable of supporting enzyme inactivation in the absence of NADPH and CPR, suggested the formation and utilization of a hydroperoxo-iron species responsible for substrate oxygenation by the T303A mutant and an iron-oxo species for use by the wild-type enzyme [401]. These results also confirmed the disruption of proton delivery to the active site in the T303A mutant [401]. One possible mechanism suggested for the reversible inactivation of CYP2E1 T303A by tBA is shown in Fig. 5.21. This scheme postulates that the inactivating intermediate is formed by insertion of an oxygen into the acetylene by a hydroperoxo-iron species. This oxygenated intermediate is responsible for the reversible loss of the enzymatic activity of the CYP2E1 mutant. This reactive intermediate can proceed by two different routes: (a) it can form an intermediate which is reversible over time and decomposes to yield the active enzyme with intact heme and with the release of an acetylene-derived carboxylic acid; or (b) the inactivating intermediate is stabilized in the presence of exogenous protons and then can result in the irreversible *N*-alkylation of the P450 heme. This second pathway is identical to the sequence of steps involved in the irreversible inactivation of the wild-type CYP2E1 by tBA. Another possible mechanism involves the addition of the oxygen to the distal carbon of the acetylene leading to formation of a complex in which the heme iron and the nitrogen are complexed as follows:

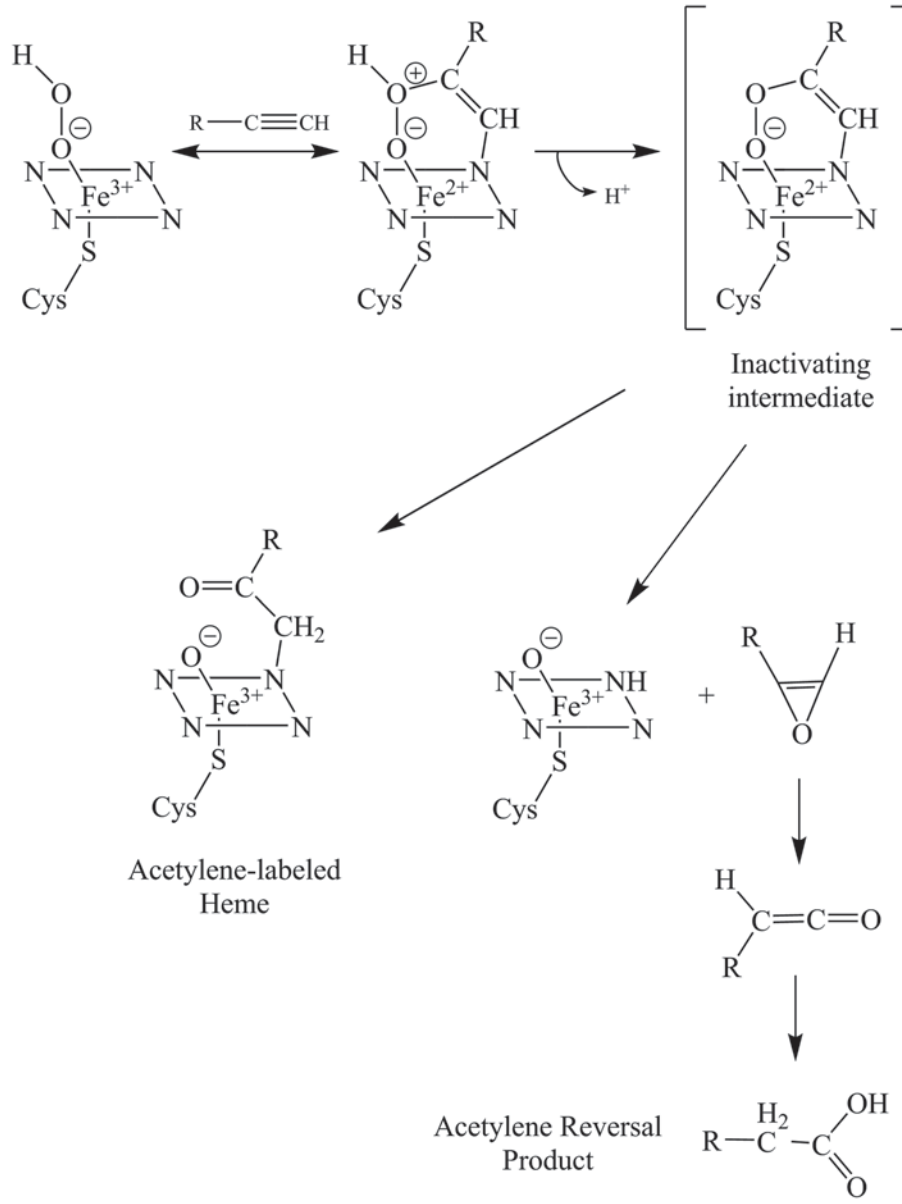


Fig. 5.21 Sequence of reactions for the reversible inactivation of CYP2E1T303A by low molecular weight acetylenes. In the initial step, the hydroperoxy-iron species in the T303A mutant inserts an activated oxygen into the acetylenic compound to form an inactivating intermediate (in brackets) that can readily be observed spectrally at 485 nm. This intermediate is responsible for the losses in enzymatic activity of the CYP2E1 mutant and its formation can either be reversed over time to regenerate the native heme and one or more reversal products or the intermediate can then *N*-alkylate the P450 heme in the presence of exogenous protons and irreversibly modify the enzyme as seen with the wild-type 2E1 enzyme [401]

Fe–O–CR=CH–N. The disruption of the complex would be promoted by acid. The primary difference between the mechanism depicted in Fig. 5.21 and the second mechanism is that the distal carbon of the acetylene is connected to the

iron by the ferryl oxygen rather than by a two-oxygen peroxide bridge.

Since Thr303 is very highly conserved in the P450 enzymes and it is thought to be involved in proton delivery to the P450 active sites, the role of this conserved residue and the protein relay networks in the reversibility of the MBI by acetylenes was examined in CYP2B4 and its T302A mutant, which corresponds to the T303A mutant in CYP2E1 [402, 403]. These studies showed that the same acetylenic inactivators (tBA and tBMP) could inactivate these two P450s in a mechanism-based manner and formed acetylene adducts with the heme [402, 403]. The inactivations of CYP2B4 and its T302A mutant were only partially reversible (20–30%) by dialysis or spin column gel filtration. The formation of the stable tBA or tBMP heme adducts in both the wild-type and mutant CYP2B4s required protons, a significant deviation from what was observed with CYP2E1 and its mutant. Models of the active site of CYP2B4 and the mutant based on the CYP2B4 crystal structure showed that its T302A mutation has no significant effect on the architecture of the enzyme active site or on the proton delivery networks, as seen with CYP2E1. There were two possible networks for proton delivery in the CYP2B4 P450s. However, the glutamate (E301) and threonine (T302) network is intact in the T302A mutant of CYP2B4. This suggests that delivery of the protons in the mutant is still efficient. Based on mass spectral data and computational modeling, it appears that the conserved Thr residue in CYP2B4 is not involved in proton delivery to the acetylene reactive intermediate in the heme or in the partial reversibility that is observed with the CYP2B4 enzymes. Therefore, these studies suggest that the active site architecture and proton relay system may play an important role in determining the reversibility of these two P450s. Models of the CYP2B4 T302A mutant reveal the presence of a compensatory ordered hydrogen bond network even in the absence of the Thr302. These results indicate that although Thr302 may play a role in proton delivery in the formation of the oxenoid-iron complex and also in the stabilization of the acetylene heme adducts in CYP2B4, it is not essential for proton delivery given the presence of Glu301 in the substrate binding site and that the conserved Glu301 may be operational in the hydrogen bond network even when the conserved Thr302 residue is absent [402, 403].

Studies on the MBI of CYP2B1 wild-type (WT; Fig. 5.22) and its T205A mutant by tBPA and *tert*-butyl 1-methyl-2-propynyl ether (*t*BMP), two structurally related *tert*-butyl acetylenic compounds showed that they inactivated CYP2B1 by two very distinct mechanisms and that the efficiencies varied by > 70-fold [404]. tBPA inactivated CYP2B1 (WT) with a K_I 0.7 μM and a k_{inact} of 1.64 min^{-1} and the T205A mutant with $K_I = 16$ and k_{inact} of 0.36. The partition ratios for the WT and mutant were 1 and 9, respectively. BMP inactivated the WT with a K_I of 17 μM and k_{inact} of 0.56 min^{-1} and the mutant with a K_I of 16 μM and k_{inact} of 0.14 min^{-1}. The partition ratios for the WT and mutant were 10 and 35, respectively. LC–MS/MS of the WT demonstrated that its inactivation by tBPA resulted in the formation of a protein adduct having a mass increase equivalent to the mass of the tBPA plus one oxygen atom and that the inactivation by BMP led to the formation of multiple heme adducts without protein adduction and that all of the heme adducts had mass increases equivalent to BMP plus one oxygen atom. Trapping of the reactive intermediates with GSH followed by LC–MS/MS analysis revealed the formation of conjugates resulting from the reaction of the ethynyl moiety of the BMP or tBPA with the oxygen being added to the internal carbon of BMP and the terminal carbon of BPA. Inactivation of the T205A mutant by BMP led to the formation of only one major heme adduct. These results demonstrate that Thr205 in the F-helix plays an important role in the efficiency of the MBI of CYP2B1 by BPA and BMP. Substrate docking and homology modeling studies helped in identifying the potential role of Thr205 in hydrogen bonding interactions affecting the three-dimensional structure of the active site [404].

tBPA was also shown to be a potent mechanism-based inactivator of CYP2B4 [405]. Inactivation occurred in an NADPH- and time-dependent manner with a K_I of 0.44 μM and a k_{inact} of 0.12 min^{-1}. Interestingly, the partition ratio was

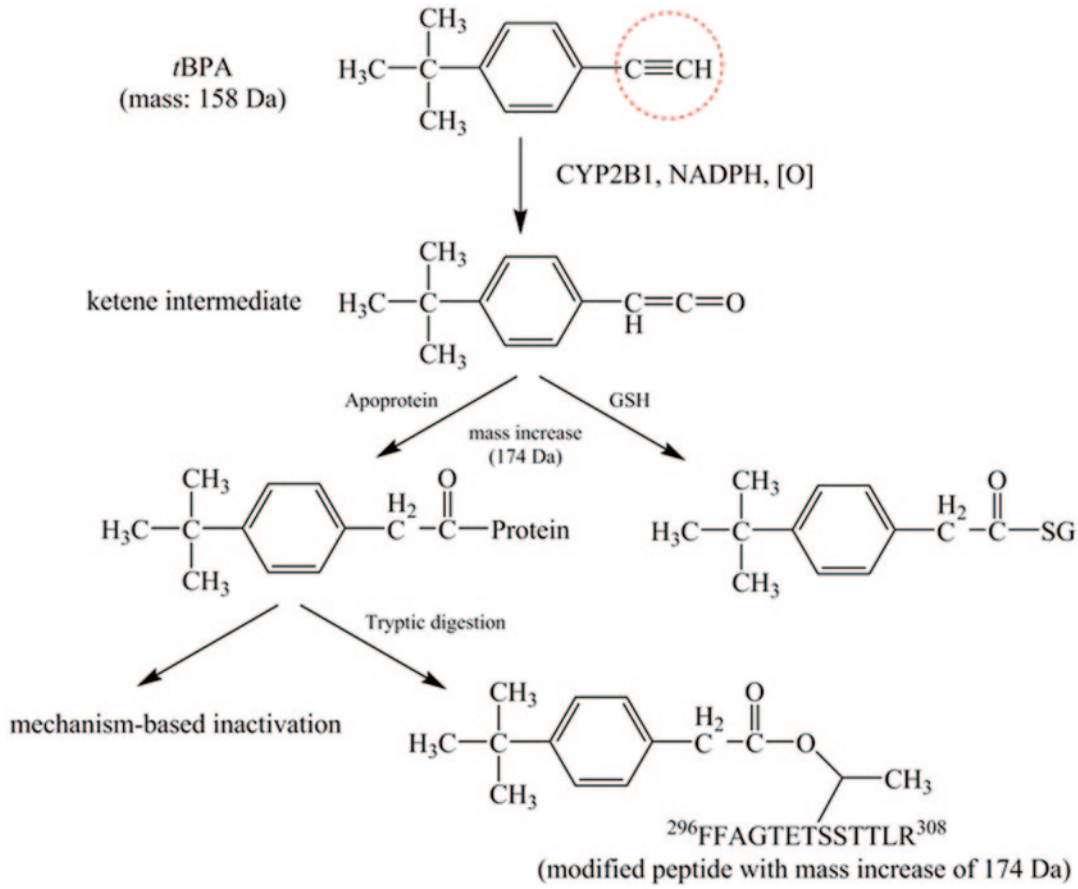


Fig. 5.22 Pathways proposed for the mechanism-based inactivation of CYP2B1 by a reactive intermediate derived from 4-*tert*-butyl phenylacetylene (*t*BPA). The ketene intermediate can react with the apoprotein leading to mechanism-based inactivation. It has also been trapped with GSH, leading to the positive identification of its structure

~0, suggesting that the inactivation occurs without any of the reactive intermediate leaving the active site. LC–MS analysis of the modified protein showed that tBPA binds to the protein with a 1:1 stoichiometry. Peptide mapping of the tBPA-inactivated CYP2B6 showed that adduct formation occurred on Thr302, consistent with molecular modeling studies showing that the terminal carbon of the acetylenic group is within 3.65 Å of Thr302. In order to investigate the effect of the formation of a covalent bond between tBPA and the CYP2B4 apoprotein at the active site, the protein was purified to homogeneity and the modified protein was characterized [405]. A red shift in the Soret peak maximum of 5–422 nm was observed with the tBPA-inactivated protein compared with the unmodified protein. Binding of benzphetamine to the inactivated CYP2B4 did not cause a spin shift, indicating that either the binding of the substrate and/or the heme environment had been altered by covalent binding and inactivation by tBPA. Although CPR reduced both the unmodified and modified P450s at the same rate, the addition of the substrate benzphetamine stimulated reduction of the unmodified CYP2B4 by ~20-fold but only marginally stimulated the rate of reduction of the tBPA-modified protein [405]. These results suggest that the impairment of the CYP2B4 catalytic activity is due to the inhibition of substrate binding to the inactivat-

ed protein. Subsequent studies using resonance Raman spectroscopy of the unmodified and the modified CYP2B4 in the absence and presence of the benzphetamine substrate demonstrated that although the modification of the protein by tBPA does not substantially alter the resting-state heme structure, it does block the entrance of the substrate to the distal pocket of the protein [406]. The results of resonance Raman spectroscopy also demonstrated that even small structural changes associated with MBI could potentially lead to significant differences in the P450 reduction potential or the affinity for its axial ligands, and also impact the stability of key hydroperoxo- or peroxo-intermediates. The fully tBPA-modified CYP2B4 was still able to catalyze the oxidation of 7-EFC, benzphetamine, and testosterone at 30, 21, and 9.6% of the rates for the unmodified CYP2B4, respectively. Thus, covalent modification by tBPA impairs the catalytic activity, but the extent of this impairment varies with the nature of the substrate probes. Therefore, even though substrate binding to the active site appears to be adversely affected by the tBPA adduct, residual activity may still arise due to the conformational flexibility of the P450 active site structure that would allow transient access of the substrates to the active site. This possibility appears reasonable because it has been previously documented that the secondary structure of CYP2B4 is very flexible [407, 408].

In order to identify the adducted residue following tBPA inactivation of CYP2B1, the modified protein was digested with trypsin and the peptides from the digest were analyzed by LC–MS/MS [409]. Based on the identification of a tBPA-GSH conjugate with an increase in mass of 174 Da and the 174 Da increase in the mass of the BPA-adducted apoprotein, a shift of 174 Da was used for a SEQUEST database search of the tryptic peptides from the CYP2B1. The tandem mass spectrometric fragmentation of the modified peptide led to identification of the modified residue. A mass increase of 174 Da for the peptide sequence ^{296}FFAGTSSTTLR^{308} in the I-helix was observed and the site of adduct formation was found to be Thr302 (Fig. 5.21). Ligand docking and homology modeling studies showed that tBPA was bound in close proximity to both Thr302 and the heme iron in CYP2B1 with the distances being 3.42 and 2.96 Å, respectively. These results support the previous hypothesis that this highly conserved Thr residue may play a crucial role in the active site of the P450s. The proposed pathways for the formation of the reactive intermediate of tBPA and the reaction to form a protein-bound adduct during tBPA MBI of CYP2B1 are shown in Fig. 5.21. It is suggested that the ketene intermediate formed by the CYP2B1-catalyzed oxidation of the acetylenic group may be oriented in the active site to facilitate nucleophilic attack by the threonine hydroxyl group leading to the formation of an ester linkage to the protein [409].

Insights into how the tBPA-modified CYP2B4 retains partial activity were obtained from a combined structural and computational analysis of the modified protein [410]. How the conjugation of the tBPA to the highly conserved Thr302 in the active site still allowed for residual activity was not clear. In order to gain a better understanding of how this occurs, the tBPA-modified CYP2B4 was crystallized and the crystal structure showed that an oxygenated metabolite of tBPA was, in fact, conjugated to the Thr302 of helix I, consistent with previous studies using LC–MS/MS. Interestingly, the modified protein crystallized in two different structural conformations. In each structure, the core of the CYP2B4 was unchanged, but the arrangement of the "plastic" regions differed. One of the structures was a compact structure in a closed conformation that was in agreement with in silico experiments that had been performed previously [410]. However, the other structure, referred to as the open structure, was formed by dimerization of CYP2B4 due to movement of the B/C loop and helixes F through G. This alters the position of tBPA so that it is almost parallel to the heme plane. Docking experiments using this open form demonstrated that the tBPA is able to rotate upward to give substrates such as 7-EFC and testosterone access to the heme, which could explain the partial retention of the catalytic activity that was observed previously.

The ability of tBPA to cause MBI of CYP2B6 was also investigated [411]. tBPA was shown to be a time-, concentration-, and NADPH-dependent inactivator of the P450. It exhibited a K_I of 2.8 μM, a k_{inact} of 0.7 min^{-1} and a partition ratio of ~5. The mass increases for a conjugate trapped with GSH and for the adducted protein were 174 Da, the same as with CYP2B1, which is equivalent to the mass of one molecule of tBPA plus one oxygen atom. The identity of the adducted residue was determined by digesting the BPA-inactivated CYP2B6 with trypsin and then analyzing the digest by LC–MS/MS. A mass shift of 174 Da was used in the SEQUEST database search and the modified residue was identified by MS/MS fragmentation of the modified peptide. Two residues, Thr302 and Lys274 were identified as the tBPA-modified residues. Subsequent mutagenesis studies demonstrated that the Thr302 was the residue that was modified leading to the inactivation, not Lys274 [411]. In order to test the experimental results, the tBPA was docked into the active site of the crystal structure of a CYP2B6 genetic variant. The active site residues within 4 Å of the reversibly bound tBPA, as well as the distance between the heme iron and the two residues suggested to be adducted by SEQUEST search were examined. The results of these docking studies agreed with the mutagenesis results, which revealed that Thr302 and not Lys274 was the critical residue modified by the tBPA reactive metabolite, this modification being responsible for the MBI [411].

Further insights into the structural and functional relationships of the P450s can be gained from molecular modeling studies. The potent inactivation by tBPA of the CYP2Bs appears to be due to its unique binding mode in the CYP2B active site s. The close proximity of the terminal carbon of the acetylenic group of tBPA to both the Oγ atom of Thr302 and the heme Fe greatly facilitates the formation of the reactive ketene intermediate and its subsequent reaction to form a covalent linkage with the hydroxyl group of Thr302. The extremely small partition ratio suggests that the ketene intermediate has a very low probability of escaping from the active site when its formation occurs in such a close proximity to the Thr302 Oγ and therefore it is a very efficient inactivator that minimizes the "collateral" damage to other cellular proteins [411].

17α-Ethynylestradiol (EE; Fig. 5.19) has been known for a long time be a mechanism-based inactivator of P450s [314–316]. The inactivations were shown to be due to activation of the acetylenic moiety to a reactive intermediate that then alkylated a pyrrole nitrogen on the heme. Studies using purified CYP3A4 in the reconstituted system demonstrated that EE was a potent mechanism-based inactivator which modified both the heme and the protein [412]. The inactivation of CYP3A4 followed pseudo-first-order kinetics and was dependent on NADPH, time, and concentration. The values for the K_I and k_{inact} were 18 μM and 0.04 min^{-1}, respectively. The partition ratio was ~50. The binding stoichiometry was ~1.3 nmol of EE per nmol of inactivated P450. SDS–PAGE demonstrated that the radiolabeled EE was irreversibly bound to the apoprotein. HPLC analysis demonstrated that the inactivation led to the destruction of approximately half the heme with the concomitant generation of modified heme and EE-labeled heme fragments, and also produced radiolabeled CYP3A4 apoprotein [412].

17EE (Fig. 5.19) was also shown to inactivate purified rat CYP2B1 and human CYP2B6 in a mechanism-based manner [413]. For CYP2B1 the K_I was 11 μM and the k_{inact} was 0.2 min^{-1}, and for CYP2B6 the K_I was 0.8 μM and the k_{inact} was 0.03 min^{-1}. Inactivation of CYP2B1 by 17EE led to approximately 75% loss in enzyme activity with a concurrent 20–25% loss in the ability to form a reduced CO complex after 20 min of incubation. With CYP2B6, a 20-min incubation led to 83% loss of enzymatic activity with only a 5–10% loss in the CO-reduced spectrum. The partition ratios for the inactivation of CYPs 2B1 and 2B6 were 21 and 13, respectively. The stoichiometry of binding of the radiolabeled 17EE to both P450s was ~1.3:1. Analysis of the metabolites of 17EE formed by all four CYP2B enzymes under investigation showed that the CYPs 2B2 and 2B4, which were not inactivated by 17EE, differed primarily in their ability to generate two metabolites, which presumably may

be products formed from the reactive intermediate responsible for the MBI [413]. It is of interest that although CYP2B2 and CYP2B4 share > 70 % sequence identity with CYP2B1, they were only minimally affected by 17EE-incubation in the presence of NADPH.

Although heme destruction was the primary cause for the inactivation of CYP3A4, there was minimal loss or modification of the heme moiety when CYP2B1 or CYP2B6 were inactivated by 17EE. Therefore, it appeared that the reactive intermediate formed from 17EE was modifying the apoprotein. Mass spectral analysis of 17EE-inactivated CYP2B1 showed an increase in the mass of the apoprotein of ~313 Da, consistent with the mass of 17EE plus one oxygen atom [414]. CNBr digestion of the radiolabeled P450s led to the identification of one major labeled peptide for each enzyme. *N*-terminal sequencing of these peptides yielded amino acid sequences that corresponded to the amino acids P_{347}-M_{376} and P_{347}-M_{365} in CYP2B1 and CYP2B6, respectively. ESI–LC–MS and MALDI-MS analysis of the CYP2B1-derived peptide resulted in a mass of 3654 Da, which is consistent with the mass of the P_{347}-M_{376} peptide (3385 Da) plus a 268-Da adduct from 17EE. GSH added to the reaction mixture was used to trap chemically reactive intermediates of 17EE generated during the MBI of the P450s. ESI–LC–MS/MS analysis of the trapped GSH conjugates from the incubation mixtures revealed that the two P450s generated different reactive intermediates of 17EE that were responsible for the formation of the adducts with the proteins, the P450 inactivation, and the formation of the GSH conjugates [414].

17EE was also shown to inactivate CYP3A5 [415]. This inactivation was dependent on b_5. The values for the K_I and k_{inact} were 26 μM and 0.06 min^{-1}, respectively. The partition ratio was ~25. The stoichiometry for binding of EE was ~0.3 mol/mol of P450 inactivated. SDS–PAGE demonstrated that radiolabeled EE was irreversibly bound to the apoprotein. LC–MS/MS revealed the formation of two GSH-conjugates with *m*/*z* values of 620 that were formed only in the presence of b_5.The two conjugates were formed by reaction of GSH with the ethynyl group of the EE with the oxygen being inserted into either the terminal or the internal carbon. A heme adduct having *m*/*z* 927 and two dipyrrole adducts having *m*/*z* values of 579 were also detected by LC–MS/MS analysis. These results suggested that CYP3A5 activates 17EE to a 17α-oxirene-related reactive species that can partition the oxygen between the terminal and internal carbons of the ethynyl group leading to the formation of both heme and apoprotein adducts that inactivate CYP3A5 [415].

Sequence alignment of CYPs 2B1, 2B2, 2B4, and 2B6 between the P_{347} and the amino acid residue at position 376 exhibited significant conservation across the four enzymes [416]. However, the single nucleophilic residue that is identical in CYPs 2B1 and 2B6, but different in CYPs 2B2 and 2B4, is S_{360}. This residue in CYP2B4 is located at the C-terminal end of the K helix and is thought to be in SRS 5. Thus, it is conceivable that the residue at position 360, particularly when present as a serine, may have an important role in the metabolism of larger molecules such as steroids. Interestingly, S_{360} is the only residue identical in both CYP2B1 and 2B6 (the isozymes inactivated by 17EE) and could form an ester linkage with a reactive intermediate of 17EE [415]. The amino acid at position 360 of CYPs 2B2 and 2B4 (the enzymes that were not inactivated by 17EE) is a glycine or alanine, respectively. These residues would not be able to form an adduct with the reactive intermediate of 17EE and thus would not be expected to be targets leading to the inactivation.

CYPs 2B1 and 2B6 were inactivated with 17EE and digested with trypsin [416]. Adducted peptides having mass increases of 312 Da, consistent with the addition of the mass of 17EE reactive intermediate were identified for each of the isozymes. ESI–MS/MS analysis of the modified peptides and precursor ion scanning led to the identification of Ser360 in both enzymes as the amino acid residue that had been modified by a reactive metabolite of 17EE. A CYP2B1 mutant in which Ser360 was replaced with alanine was constructed, expressed, and purified [417]. Interestingly, this mutation did not prevent inactivation by 17EE. However, it did cause

a significant change in the inactivation kinetics by 17EE, as well as altered the product profile formed from testosterone. Spectral binding studies of the 17EE-inactivated CYPs 2B1 and 2B6 indicated that this modification resulted in an enzyme that no longer exhibited a binding spectrum. These results suggest that the 17EE inactivation of CYPs 2B1 and 2B6 may be due to the modification of an amino acid residue either in the substrate access channel or near the point of entry into the active site that is not critical for the catalytic function of the P450s, but is in the vicinity of the substrate binding and its modification can play a significant role in altering the binding orientation of large substrates such as steroids [416]. The residues at positions 363 and 367 in CYP2B4 have been shown to be within 5 Å of the ligand bound in the active site, and residue 363 plays a functional role in steroid metabolism. Examination of the crystal structure of CYP2B4 shows that the S_{360} residue is not located within 5 Å of the heme, but may occupy a position in the access channel to the heme. Therefore, the loss in function of the P450s by covalent modification of S_{360} is probably not a consequence of the catalytic role of this residue, but is more likely to be due to steric hindrance by blocking substrate access to the active site [416].

Deprenyl (Fig. 5.19) is a propargylamine having a terminal acetylenic group and it has been shown previously to be a mechanism-based inactivator of the MAO via covalent modification of the MAO flavin moiety [417]. Deprenyl has also been shown to inactivate CYP2B1 with a K_I of 1.05 μM, a k_{inact} of 0.23 min^{-1}, and a partition ratio of ~2 [418]. Although a loss in the spectrally detectable P450 chromophore was observed, there was no significant change observed in the heme absorbance at 405 nm. These results were interpreted as suggesting that protein modification rather than heme modification was involved. Selegiline, the *R*-enantiomer of deprenyl which is used in the treatment of Parkinson's disease, was also shown to be a mechanism-based inactivator of human CYP2B6 [419]. It was a mechanism-based inactivator of 7-EFC activity and the oxidative metabolism of bupropion. The inactivations were time-, concentration-, and NADPH-dependent [419]. The K_I values were 0.14 and 0.6 μM, the k_{inact} values were 0.022 and 0.029, respectively. Although there was a significant decrease in the reduced CO-difference spectrum, there was no loss in the heme content of the proteins. GSH trapping of the reactive intermediate resulted in the identification of a GSH-selegiline conjugate with a *m/z* 528 that could be explained by the hydroxylation of selegiline followed by the addition of GSH to the propargyl moiety after oxygenation leading to the formation of the ketene intermediate. LC–MS/MS analysis of the peptides following digestion of the labeled protein with trypsin revealed the peptide ^{64}DVFTVHLGPR^{73} as the peptide that had been modified by the reactive metabolite of selegiline and the site of adduct formation as Asp64 [419].

5.3.3.3 Other Inactivators That Modify the P450 Proteins

Phencylidine (PCP; Fig. 5.23) has been shown to be a mechanism-based inactivator of CYPs 2B1 and 2B6 [420, 421]. The inactivations of both P450s were time-, concentration-, and NADPH-dependent and exhibited pseudo first-order kinetics. Since there was no loss in spectrally detectable heme, it was concluded that the inactivation involved covalent binding of a reactive intermediate of the PCP to the apoprotein. The mass difference between the unmodified and the PCP-inactivated P450s was 244 Da, which corresponds to the binding of one mol of PCP per mol of P450 inactivated. Five major metabolites of PCP were identified, including a product derived from hydroxylation on the piperidine ring as shown in B of Fig 5.23. The hydroxylation of the piperidine ring appears to be the primary reaction responsible for the formation of the reactive intermediate leading to the inactivation reaction. P450s 2B1 and 2B4 formed a novel metabolite having an m/z of 240 which corresponds to the expected mass for the 2,3-diihydropyridinium species of PCP. GSH- and *N*-acetylcysteine (NAC)-trapping studies also resulted in the formation of conjugates that were consistent with the mass of a 2,3-dihydropyridinium ion. These data suggest that the reactive intermediate is the enamine formed following oxidation

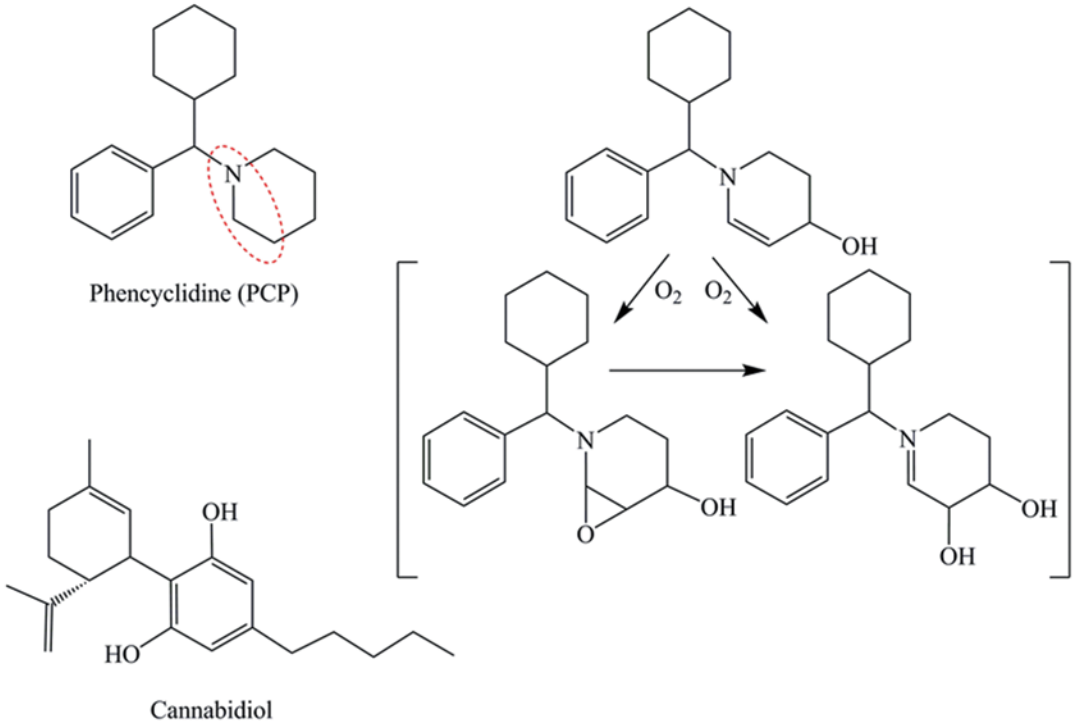


Fig. 5.23 **a** Structure of phencyclidine (PCP). The circled area indicates the site of metabolism leading to the formation of the reactive intermediate; **b** Pathway for formation of the proposed reactive intermediates of PCP: **c** Structure of cannabadiol

of the α-carbon of the piperidine ring to generate the iminium ion. The iminium ion has previously been proposed as the reactive intermediate. However, this finding together with the fact that NADPH was required for the inactivation of P450s by the iminium ion ruled out the iminium ion as the inactivating species [420, 421]. Human CYP2B6 formed a completely different reactive intermediate that corresponded to a dioxygenated species that could be trapped as a GSH- or NAC-conjugate. This reactive intermediate may have been generated by CYP2B6 from the enamine intermediate by oxidation of the piperidine at the 4-position followed by a second hydroxylation at the three-position or possibly by the formation of a 3,4-epoxide as shown in Fig 5.23b.

Cannabidiol (CBD; Fig. 5.23) has been shown to modify the P450 protein. CBD is a major constituent of marijuana and its ability to inactivate P450s may play an important role in its activity. The inactivation of mouse P450 isozymes 2C and 3A by CBD occurs via stoichiometric covalent binding of the inhibitor to the proteins [422, 423]. GSH-trapping of the reactive intermediate formed from CBD identified a CBD-hydroxyquinone as the inactivating species [423]. LC–MS/MS analysis of the proteolytic digest of the CBD-inactivated CYP3A11 led to the identification of two labeled peptides spanning residues A344-K379 and G426-K454. These regions correspond to SRS-5 in the K-region of the CYP3A11 active site and the heme-binding Cys443-region of the helix L domain [389–391]. Both peptides contain Cys residues that might possibly react with the CBD-hydroxyquinone.

The furanocoumarins are another class of compounds that have been shown to inactivate rat and human liver P450s via modification of the apoprotein [424–426]. Furanocoumarins such as bergamottin (BG), 8-methoxypsoralen (8-MOP) and 8-geranyloxypsoralen (Fig. 5.24) are found as components in many foods and have

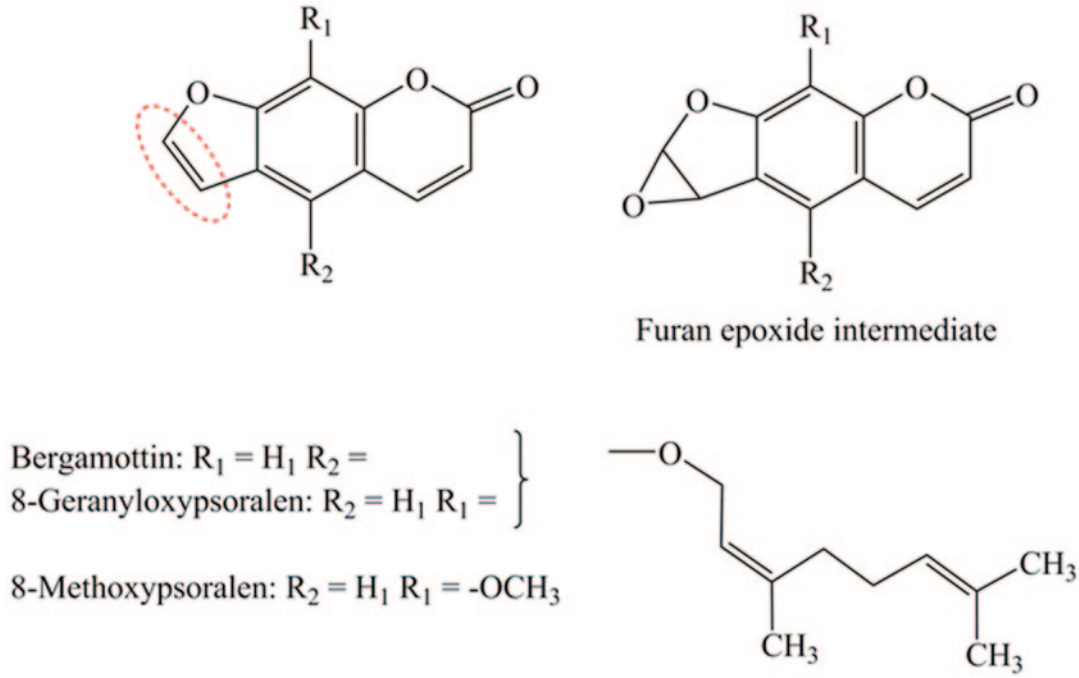


Fig. 5.24 Furanocoumarin-mediated P450 inactivation. Structures of the furanocoumarins bergamottin (BG), 8-geranyloxypsoralen, 8-methoxypsoralen (8-MOP), and the reactive furan epoxide intermediate formed from each of these compounds. The circled area indicates the site of metabolism leading to the reactive epoxide

been shown to inhibit xenobiotic metabolism. BG, one of the components responsible for the "grapefruit juice effect" has been shown to be a mechanism-based inactivator of CYPs 2B1, 2B4, 2B6, 3A4, and 3A5 [425, 426]. The inactivations of CYPs 2B6 and 3A5 were time-, concentration-, and NADPH-dependent. The kinetic constants for the inactivation of CYP2B6 were: K_I of 5 μM and a k_{inact} of 0.09 min^{-1}. For CYP3A5 they were: K_I of 20 μM and k_{inact} of 0.45 min^{-1}. The partition ratios for CYPs 2B6 and 3A5 were ~2 and ~20, respectively. SDS-PAGE analysis demonstrated that radiolabeled BG was irreversibly bound to the apoprotein of the BG inactivated enzymes. The stoichiometry of binding was ~0.5 mol of BG metabolite/mol of each P450 inactivated. HPLC analysis of the reaction mixtures indicated that CYP2B6 generated two major metabolites of BG, whereas CYP3A5 generated those two and an additional three. Two of the metabolites were identified as bergaptol and 6′,7′-dihydroxybergamottin [425]. ESI–LC–MS analysis of CYPs 2B1, 2B4, 2B6, and 3A5 inactivated by BG in all cases resulted in an increase in the mass of the apoprotein by 388 Da. This suggests that BG may first be metabolized to give the 6,7′-dihydroxy BG followed by the addition of one oxygen to the furanocoumarin moiety to form a reactive epoxide intermediate. This intermediate could then react with a nucleophilic residue in the P450. The metabolic pathway resulting in the production of a reactive intermediate of BG that could inactivate the P450s was investigated [426]. BG was metabolized primarily by CYP2B6 to give two major metabolites, 5′-OH-BG and a mixture of the 6′- and 7′-OH-BG, with bergaptol formed as a relatively minor metabolite. BG metabolism by CYP3A5 resulted in three major metabolites: 2′-OH-BG and 5′-OH-BG, bergaptol, and two minor metabolites, 6′,7′dihydroxy-BG and the mixture of 6′- and 7′-OH-BG.GSH-trapping of the reactive intermediates formed from BG by CYPs 2B6 and 3A5 followed by LC–MS analysis indicated that

the conjugates exhibited *m*/*z* values of 662 Da. MS/MS analysis of these conjugates indicated that the oxidation that led to the formation of the reactive intermediate occurred on the furan moiety, presumably through initial addition across the furan double bond to give an epoxide. In order to identify the residue on the apoprotein modified by the reactive metabolite of BG, the inactivated CYP3A4 was digested with trypsin and the digests were analyzed by LC–MS/MS. A search of the SEQUEST database was performed using a mass shift of 388 Da. A modified peptide having a mass increase of 388 Da was identified having the sequence ^{272}LQLMIDSQNSK^{282}. MS/MS analysis of this peptide demonstrated that Gln273 was the residue modified. Mutagenesis studies in which the Gln273 was mutated to a Val showed that the mutant protein was resistant to inactivation by both BG and the DHBG [427]. Under the same conditions, LC–MS/MS analysis of BG-inactivated CYP3A5 demonstrated covalent modification of Gln273 during BG inactivation. Analysis of the CYP3A4 crystal structure shows that Gln273 is actually far away from the heme iron (~20 Å) and is not in the active site. However, the hydrogen bonding distance between the Gln273 amine group and the Asp277 carboxylate side chain is 2.4 Å. Thus, it was proposed that covalent formation of an amide bond between the NH_2 group of Gln273 and the furanoepoxide of DHBG would disrupt this hydrogen bond interaction, thereby compromising the formation of the preferred secondary and tertiary structures of CYP3A4, resulting in impaired catalysis [427].

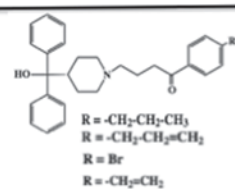
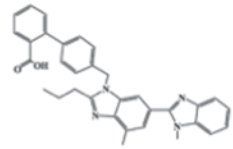
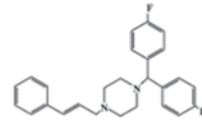
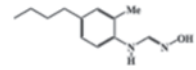
8-MOP (Fig. 5.24) has been shown to be a potent mechanism-based inactivator of CYPs 2A6, 2A13, 2B1, 2B2, 2C11, and 3A [428–431]. 8-MOP contains the same furanocoumarin core structure as BG. Of all of the furanocoumarins that have been tested on CYP2B1, 8-MOP was the most potent with a K_I of 2.9 μM, a k_{inact} of 0.34 min^{-1}, and a partition ratio of 1.3 [428]. HPLC or SDS–PAGE analysis of incubations of the purified CYP2B1 with radiolabeled 8-MOP showed that the radiolabel was bound to the protein rather than the heme and the binding stoichiometry was 0.7:1. LC–ESI–MS analysis of the modified CYP2B1 revealed a mass shift of 237.9±9.6 Da for the modified enzyme. Similar studies for the psoralen- and 5-MOP-modified CYP2B1 gave mass shifts of 204±11.8 Da and 240±6.2 Da, respectively [428]. These results indicate that a single molecule of psoralen is covalently bound to the protein. The steps in generating the reactive intermediate that bound to the protein require an initial epoxidation reaction followed either by hydrolysis or attack by a nucleophile to form the dihydrofuranocoumarin products. As with BG, the furanepoxide is considered to be the key reactive intermediate responsible for the P450 modification and inactivation [428].

L-754,394, *N*-[2(*R*)-hydroxy-1(*S*)-indanyl]-5-[2(*S*)-((*1,1*-dimethylethyl)*amino*)carbonyl]-4-[(furo[2,3-b]pyridin-5-yl)methylpiperazin-1-yl]-4(*S*)-hydroxy-2(*R*)(phenylmethyl)-pentenamide, a furanopyridine, is also a potent mechanism-based inactivator of human CYP3A4 as well as human CYP2D6 [432–435]. For the inactivation of CYP3A4 the K_I was 7.5 μM, the k_{inact} was 1.62 min^{-1}, and the partition ratio was 1.35 [433]. Identification of the metabolites generated during metabolism of the L-754,394 indicated that the mechanism of inactivation probably involves oxidation of the furan ring to the corresponding epoxide and/or γ-ketoenal that binds to the CYP3A4 protein at its active site. Attempts to isolate the adducted peptide using proteolytic or CNBr digestion were unsuccessful, demonstrating the labile nature of the peptide adduct and precluding direct identification of the covalently modified amino acid or the peptide to which it was attached. However, Tricine SDS-PAGE was used in combination with MALDI-TOF-MS and homology modeling to tentatively identify the peptide spanning residues I257-M317 as the active site peptide. Based on the knowledge of the stability of *N*-, O-, and *S*-linked conjugates of activated furans, the authors suggested that Glu307 was the active site amino acid that was labeled leading to the inactivation [434].

5.4 Therapeutic Exploitation of P450 Inhibitors

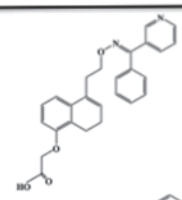
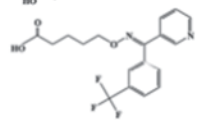
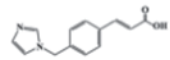
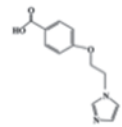
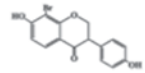
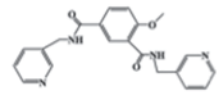
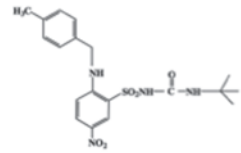
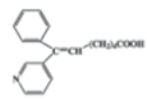
The various inhibitory structural features discussed in the text have been very aptly exploited in the therapeutic development of chemical in-

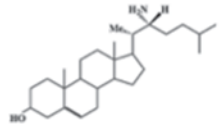
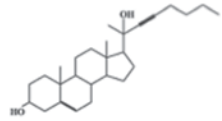
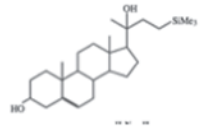
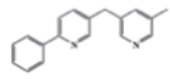
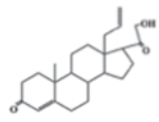
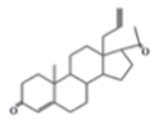
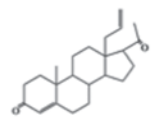
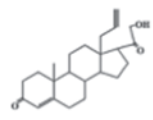
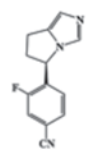
Table 5.1 Some notable inhibitors of therapeutically relevant pathophysiologic or parasitic P450s

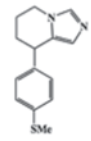
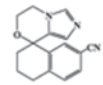
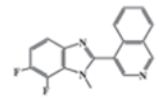
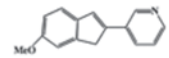
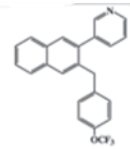
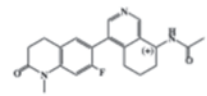
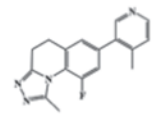
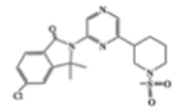
P450 target	Inhibitor	Structure	Inhibition Mode[a]	Therapeutic or Investigational use[b]	Reference
CYP2J2	Terfenadine-analogs	 R = -CH_2-CH_2-CH_3 R = -CH_2-CH_2=CH_2 R = Br R = -CH_2=CH_2	C TDI/MBI C TDI/MBI	Anti-neoplastic	*436-438*
	Telmisartan		Mixed Type	Diagnostic Probe	*439*
	Flunarizine		C	Diagnostic Probe	*439*
CYP4A11	HET0016		C	Diagnostic Probe	*440*
	17-ODYA		MBI	Antihypertensive?	*441-452*
	DDMS		C	Antihypertensive?	*67, 453,454*
	10-SUYS		MBI	Diagnostic Probe	*455*
	MSPPOH		MBI	Diagnostic Probe	*456-458*
CYP4V2	HET0016		C	Diagnostic Probe	*459*

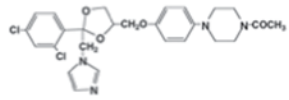
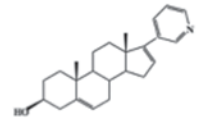
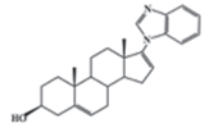
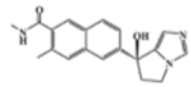
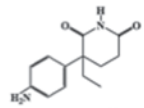
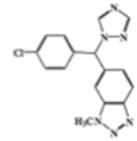
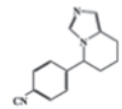
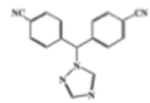
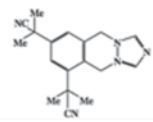
hibitors targeted against human and parasitic P450s of pathological relevance. A concise list of such prototypic chemical inhibitors of some biosynthetic P450s and/or pathophysiologically relevant P450s that are clinically established drugs, drugs currently in clinical trials, or prospective drug candidates, or even agents that may be used experimentally as diagnostic probes of a given P450, is provided (Table 5.1). A more comprehensive literature coverage of P450 enzyme inhibitors follows in Chap. 9 by F. P. Guengerich.

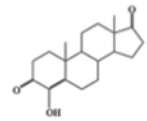
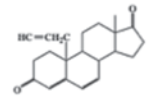
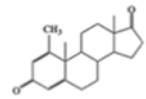
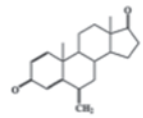
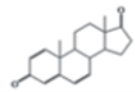
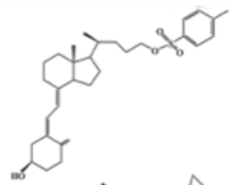
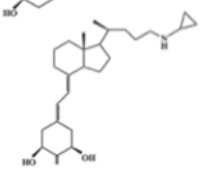
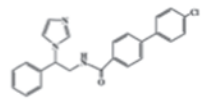
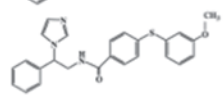
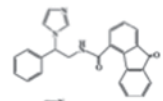
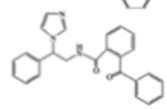
Acknowledgments We gratefully acknowledge the generous contributions of the structures in Fig. 2B by Dr. Tove Sjogren and in Fig. 2C by Prof. Emily E. Scott. M. A. Correia's contribution to this chapter was supported by NIH grants GM44037 and DK26506, and that of P. F. Hollenberg by NIH grant CA16954.

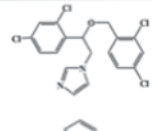
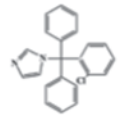
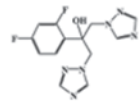
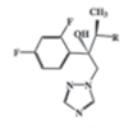
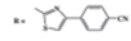
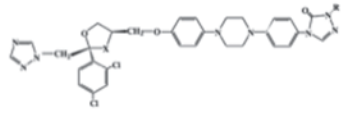
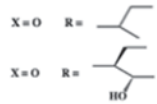
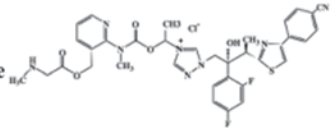
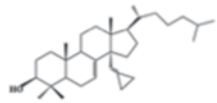
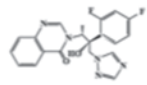
P450 target	Inhibitor	Structure	Inhibition Mode	Therapeutic or Investigational use	Reference
CYP5A1					
	ONO-0131		**C**	**Pro-angiogenic Anti-HCV replication**	*460, 461*
	Ridogrel		**C**	**Anti-platelet aggregation**	*462, 463*
	Ozagrel		**C**	**Asthma**	*463-467*
	Dazoxiben 4-[2-(1H-imidazol-1-yl) ethoxy]benzoic acid		**C**	**Anti-thrombotic**	*463, 467*
	Nv-52		**C**	**IBD[c]**	*468*
	Picotamide		**C**	**Peripheral artery disease**	*469-471*
	Azo analog I (9,11-azoprosta-5,13-dienoic acid)		**C**	**Anti-platelet aggregation**	*472*
	BM-573		**C**	**Anti-thrombotic Atherosclerosis**	*473, 474*
	CGS-13080		**C**	**Anti-platelet aggregation Diagnostic probe**	*475*
	CV-1451		**C**	**Diagnostic probe**	*476*
	EV-077	***Yet to be divulged***	**C?**	**Anti-platelet aggregation**	*477, 478*

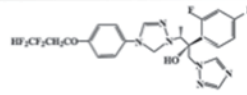
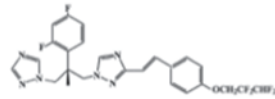
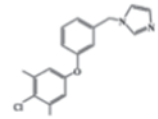
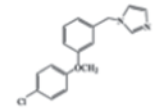
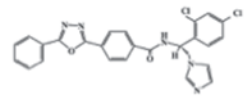
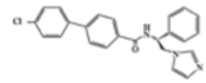
P450 target	Inhibitor	Structure	Inhibition Mode	Therapeutic or Investigational use	Reference
CYP11A1					
	(22R)-22-Amino-cholesterol		**C**	**Probe**	*479*
	20-(1-hexynyl)-5-pregnen-3β,20α-diol)		**MBI**	**Probe**	*480-482*
	20-(2-(Trimethyl-Silyl)ethyl-20-5-pregnen-3β,20α-diol)		**C**	**Probe**	*483*
CYP11B1					
	#44 (5-((1*H*-imidozol-1-yl)methyl)-2-phenylpyridine)		**C**	**Cushing's syndrome?**	*484*
	18-Vinyl-DOC		**MBI**	**Probe**	*485*
	18-Ethinylprogesterone		**MBI**	**Aldosterone reduction**	*486*
	18-Vinylprogesterone		**MBI**	**Aldosterone reduction**	*486*
	18-Ethinyldeoxy corticosterone (DOC)		**MBI**	**Aldosterone reduction**	*485, 487*
	LCI699 (R)-4-(6,7-dihydro-5h-pyrrolo-[1,2-c] Imidazol-5-yl)3-fluorobenzonitrile		**C**	**Aldosterone reduction**	*488 & references therein*

P450 target	Inhibitor	Structure	Inhibition Mode	Therapeutic or Investigational use	Reference
CYP11B2 (Cont'd)					
	#10 Fadrozole derivative		**C**	**Aldosterone reduction**	*488 & references therein*
	#11 Fadrozole derivative		**C**	**Aldosterone reduction**	*488 & references therein*
	#14 Heterocyclic benzimidazole derivative		**C**	**Aldosterone reduction**	*488 & references therein*
	#21 Heterocycle-substituted indene derivative		**C**	**Aldosterone reduction**	*488 & references therein*
	#25 Disubstituted benzyl-pyridinyl naphthalene-derivative		**C**	**Aldosterone reduction**	*488, 489, & references therein*
	#33 Acetamido-substituted tetrahydroisoquinolino-derivative		**C**	**Aldosterone reduction**	*488 & references therein*
	#34 3-Pyridyl-substituted 4,5,-dihydro[1,2,4]triazolo (4,3-*a*] quinoline		**C**	**Aldosterone reduction**	*488 & references therein*
	#37 Bicyclic dihydroisoquino-line-1-one derivative		**C**	**Aldosterone reduction**	*488 & references therein*

P450 target	Inhibitor	Structure	Inhibition Mode	Therapeutic or Investigational use	Reference
CYP17A1					
	Ketoconazole		**C**	**Prostate cancer**	*105, 106*
	Abiraterone		**MBI**	**Prostate cancer**	*486-495*
	VN124-1 (TOK-001) 3-β-hydroxy-17-(1H-benzimidazol-1-yl)androsta-5,16-diene		**MBI**	**Prostate cancer**	*496*
	Orteronel (TAK-700)		**C**	**Prostate cancer**	*497-499*
	Aminoglutethimide		**C**	**Breast cancer**	*500-502*
	R-76713		**C**	**Breast cancer**	*503-505*
	Fadrozole (CGS16949A)		**C**	**Breast cancer**	*121, 123 505-510*
	Letrozole (Femara; CGS 20267)		**C**	**Breast cancer**	*122 510-512*
	Anastrozole (Arimidex)		**C**	**Breast cancer**	*513-515*

P450 target	Inhibitor	Structure	Inhibition Mode	Therapeutic or Investigational use	Reference
CYP19 (Contd.)	**Formestane** (**4-OHA;** 4-hydroxy-4-androstene-3,17-dione)		MBI	Breast cancer	*516-518*
	MDL 18962		MBI	Breast cancer	*519-525*
	Atamestane (**SH489;**1-methylandros-ta-1,4-diene-3,17-dione)		MBI	Breast cancer	*525, 526*
	Exemestane (Aromasin)		MBI	Breast cancer	*527, 528*
	ATD		MBI	Breast cancer	*529, 530*
	TS17		C	Chemotherapeutic adjuvant through Vit. D salvage	*97*
	CPA1		C	Chemotherapeutic adjuvant through Vit. D salvage	*531*
	VID400		C	Chemotherapeutic adjuvant through Vit. D salvage	*532-534*
	VAB636		C	Chemotherapeutic adjuvant through Vit. D salvage	*532-534*
	SDZ-286907		C	Chemotherapeutic adjuvant through Vit. D salvage	*532-534*
	SDZ-287871		C	Chemotherapeutic adjuvant through Vit. D salvage	*532-534*

P450 target	Inhibitor	Structure	Inhibition Mode	Therapeutic or Investigational use	Reference
CYP51A1	Miconazole		C	Antifungal	*535-538*
	Clotrimazole		C	Antifungal	*537-539*
	Fluconazole		C	Antifungal	*537-540*
	Voriconazole		C	Broad-spectrum[d]	*537-540* *541-544*
	Ravuconazole		C	Broad-spectrum	*539*
	Albaconazole		C	Broad-spectrum	*543, 544*
	Itraconazole		C	Broad-spectrum	*103, 109, 110*
	Posaconazole		C	Broad-spectrum	*103, 107, 110*
	Isavuconazole		C	Broad-spectrum	*543, 544*
	MCP		MBI	Chagas Disease	*545*
	UR9825		C	Chagas Disease	*546*

P450 target	Inhibitor	Structure	Inhibition Mode	Therapeutic or Investigational use	Reference
CYP51A1 (Cont'd)	TAK187		C	Chagas Disease	*546*
	D0870		C	Chagas Disease	*546*
	NEU321		C	Chagas Disease	*547*
	NEU704		C	Chagas Disease	*547*
	VNI		C	Chagas Disease	*539, 548*
	VNF		C	Chagas Disease	*547*

[a] **C,** competitive inhibitor
TDI, time-dependent inhibitor
MBI, mechanism-based inhibitor

[b] Chapter 6 on "Human cytochrome P450 enzymes" by F. P. Guengerich provides a more comprehensive list of references for each individual P450 enzyme inhibitor.

[c] **IBD**, Inflammatory bowel disease

[d] Broad spectrum agent against both fungal and protozoan infectious pathogenic organisms such as *T. cruzei*, *T. leishmania*, *etc*.

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Microbial Cytochromes P450

6

Kirsty J. McLean, David Leys and Andrew W. Munro

6.1 Introduction

6.1.1 General Properties of Microbial P450s

The cytochromes P450 (P450s or CYPs) were discovered in mammalian tissues 50 years ago, and crucial studies from Omura and Sato, and from the Klingenberg and Estabrook groups identified their hemoprotein nature. Further studies confirmed their link to drug/xenobiotic metabolism, and the fact that these enzymes have a distinctive coordination of their heme iron. This unusual heme ligation was later shown to originate from a cysteine thiolate bond to the iron, conserved throughout P450 oxygenase enzymes [1–5]. Numerous studies on the catalytic and structural properties of human and other mammalian P450s have been highly informative on the roles of the different P450s in functions such as steroid and eicosanoid synthesis and metabolism, and in phase I metabolism of countless pharmaceuticals [6, 7]. The P450s are usually present in considerably larger numbers in mammals and higher eukaryotes compared to the prokaryotes. For example, there are 57 *CYP* genes in humans and 272 in *Arabidopsis thaliana* (including 26 pseudogenes) compared to only one in *Campylobacter jejuni* and 20 in *Mycobacterium tuberculosis* [8–11]. However, the P450 field has benefited enormously from fundamental research progress made on prokaryotic P450s, including key information on the structural composition of the P450s, and on the mechanism by which P450s activate dioxygen and oxidize their substrates [12, 13]. In lower eukaryotes there are large numbers of CYP genes (e.g., 10 in *Candida albicans* and 111 in *Aspergillus nidulans*), with crucial roles including sterol biosynthesis and the production of oxylipins (psi, or precocious sexual inducer, factors) that regulate the sexual/asexual life cycles of *A. nidulans* [14–17]. The numbers of individual CYP genes encoded in different microbial genomes, along with key roles for the P450s in these organisms, are presented in Table 6.1. This chapter describes the diversity of microbial P450s and their physiological, biomedical, and biotechnological importance. The important role that structural, biophysical, and protein engineering studies of microbial P450s has played in our current understanding of P450 function is also emphasized.

A. W. Munro (✉) · K. J. McLean · D. Leys
Faculty of Life Sciences, Manchester Institute of Biotechnology, The University of Manchester, 131 Princess Street, Manchester M4 6JA, UK
e-mail: Andrew.Munro@manchester.ac.uk

K. J. McLean
e-mail: Kirsty.Mclean@manchester.ac.uk

D. Leys
e-mail: David.Leys@manchester.ac.uk

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Table 6.1 Numbers of cytochrome (CYP) genes and characterized functions of encoded P450 enzymes in selected microbial genomes. Organisms are presented in alphabetical order with color coding: archaea—light gray, bacteria—clear, fungi—mid-gray and protists—dark gray. Common bacterial genomes with no apparent *CYP* genes include: *Clostridium difficile, Escherichia coli, Helicobacter pylori, Legionella pneumophila, Listeria monocytogenes, Salmonella typhimurium* LT2, *Sphingomonas paucimobilis,* and *Streptococcus agalactiae* NEM316

Organism	P450s	CYP families	Function(s)	PDB ID(s)
Amycolatopsis orientalis (bacteria)	15	CYP105, CYP146, CYP164–165	β-tyrosine hydroxylase (CYP146A1, OxyD), oxidative phenol coupling of CD-(CYP165B3 OxyB) and DE-(CYP165A3 OxyA) rings, and biaryl cyclization of AB-rings (CYP165C4 OxyC) in vancomycin biosynthesis [237, 238, 248, 320]; epothilone B hydroxylase (CYP105 EpbH) [299]	1LFK, 1LGF, 1LG9 (OxyB), 1UED (OxyC)
Aspergillus nidulans (fungi)	120	89 families including CYP51F1, CYP51F2, CYP56, CYP58–62, CYP65, CYP68, CYP503–505, CYP646–687	Sterol 14α-demethylase (CYP51) [740]; sterigmatocystin synthase (CYP59A1, CYP60A1, CYP62A1) [741]; phenylacetate 2-hydroxylase (CYP504A1) [518]; fatty acid isomerase in dual function P450/peroxidase fusion (PpoA, CYP6001A1) [481]	
Bacillus megaterium (bacteria)	6	CYP102A1 (BM3), CYP106A1, CYP106B1, CYP109A2, CYP109E1	Fatty acid hydroxylase (CYP102A1) [28, 271, 742]; steroid 15β-hydroxylase (CYP106) [63]	E.g., 2HPD, 1FAG, 4KFO (BM3)
Bacillus subtilis (bacteria)	8	CYP102A2, CYP102A3, CYP107H1 (BioI), CYP107J1, CYP107K1, CYP109B1, CYP134A1, CYP152A1 (BS_β)	Fatty acid hydroxylases (CYP102A) [689], (CYP152A1) [577, 743]; pulcherriminic acid synthase (CYP134A1) [393]; oxidative cleavage of ACP-linked fatty acids involved in pimelic acid synthesis (BioI) [64, 131]	3EJB, 3EJD, 3EJE (BioI), 3NC3 (CYP134A1), 1IZO (BS_β)
Campylobacter jejuni (bacteria)	1	CYP172A1 (Cj1411c)	Role in modifying bacterial surface [10]	
Citrobacter braakii (bacteria)	1	CYP176A1 (P450cin)	1,8-cineole 2-endo-monooxygenase [38, 121]	E.g., 4FB2, 4FMX (P450cin)
Candida albicans (fungi)	10	CYP51, CYP52, CYP56 CYP61, CYP501, CYP5217A1	Sterol 14α-demethylase (CYP51) [744, 745]; fatty acid hydroxylase (CYP52A21) [746]; di-tyrosine generation (CYP56) [457]; sterol Δ22-(CYP61A2) [508]; and Δ5,6-(Erg3) [747] desaturase	
Dictyostelium discoideum (slime mold, mycetozoa)	55	CYP51, CYP508, CYP513–524, CYP554–556	Sterol 14α-demethylase (CYP51); hydroxylation of chlorinated alkyl phenone: differentiation-inducing factor-I (DIF-1) [748]	
E. coli (bacteria)	0			
Fusarium oxysporum (fungi)	164	82 families including CYP51F, CYP53–54, CYP55A1 (P450nor), CYP505, CYP620–624	Nitric oxide reductase with denitrification role (CYP55A1) [327, 749]; fatty acid hydroxylase (CYP505A1) [693]; fatty acid isomerization in dual function P450/peroxidase fusion (CYP6003A1) [721]	E.g., 1ROM, 1GED, 1XQD (CYP55A1)

Table 6.1 (continued)

Organism	P450s	CYP families	Function(s)	PDB ID(s)
Mycobacterium tuberculosis (bacteria)	20	CYP51B1, CYP121, CYP123–126, CYP128, CYP130, CYP132, CYP135A1 CYP135B1, CYP136, CYP137–144	Sterol 14α-demethylase (CYP51B1) [408, 430]; C–C bond formation of cYY (CYP121A1) [65]; cholesterol and 4-cholesten-3-one oxidases (CYP125A1, CYP142A1 [and CYP124A1]) [360, 363, 364]; branched chain fatty acid hydroxylase (CYP124A1) [395]; putative menaquinone oxidase (CYP128A1) [353]	E.g., 1EA1 (CYP51B1), 1N40 (CYP121A1), 2WM5 (CYP124A1), 3IVY (CYP125A1), 2UUQ (CYP130A1), 2XKR (CYP142A1)
Mycobacterium smegmatis mc(2)155 (bacteria)	39	CYP51, CYP105, CYP107–109, CYP123–126, CYP136, CYP138, CYP140, CYP144, CYP150–151, CYP164, CYP185–191, CYP268	Cholesterol oxidases (CYP125A3 and CYP142A2) [367]; fatty acid hydroxylase (CYP164A2) [412]	4APY (CYP125A3), 3ZBY (CYP142A2), 3R9B (CYP164A2)
Mycobacterium ulcerans Agy99 (bacteria)	21	CYP51, CYP105Q4, CYP108B4, CYP123–126, CYP136, CYP142–144, CYP187–89, CYP191	Mycolactone synthase/hydroxylase (CYP140A7) [398, 402]	
Neurospora crassa (fungi)	43	39 families including CYP51F1, CYP53–55, CYP61, CYP65, CYP68, CYP505, CYP507, CYP527–560	Probable sterol 14α-demethylase (CYP51F1) and sterol 22-desaturase (CYP61) [15]	
Novosphingobium aromaticivorans (bacteria)	16	CYP101, CYP108, CYP111, CYP153, CYP196, CYP203–204, CYP219, CYP223–225	Ionone derivative hydroxylation (CYP101C1) [750]; terpenoid (camphor) hydroxylase (CYP101D1) [118]; camphor 5-exo hydroxylase (CYP101D2) [119]; aromatic hydrocarbon hydroxylase (CYP108D1) [118, 751]; alkane hydroxylase (CYP153C1) [126]	3OEU (CYP101C1), 3LXI (CYP101D1), 3NV5 (CYP101D2), 3KTK (CYP108D1)
Phanerochaete chrysosporium (fungi)	149	33 families to date including CYP51, CYP53, CYP61, CYP63, CYP5136–5158	Hydroxylation of polycyclic aromatic hydrocarbons (various CYPs, e.g., [74–77])	
Picrophilus torridus (archaea)	2	CYP231A1, CYP231A2	Orphan P450s [103]	2RFB, 2RFC (CYP231A2)
Pseudomonas fluorescens PfO-1 (bacteria)	3	CYP221A1, CYP229A1, CYP230A1	Acyl CoA dehydrogenase/P450 fusion (CYP221A1); Putative role in Mupirocin biosynthesis (CYP203A1) [752]	
Pseudomonas putida (bacteria)	2	CYP101A1, CYP111	Camphor 5-hydroxylase (P450cam) [12, 753]; linalool 8-monooxygenase (P450lin) [754, 755]	E.g., 2CPP, 1AKD, 4JWU (P450cam)
Saccharomyces cerevisiae (fungi)	3	CYP51A1, CYP57, CYP61	Sterol 14α-demethylase (CYP51A1) [467]; di-tyrosine generation for spore wall formation (CYP57) [456]; sterol 22-desaturase (CYP61) [453]	4KOF, 4LXJ (CYP51A1 with membrane-spanning helix)
Saccharopolyspora erythraea NRRL23338 (bacteria)	36	Including CYP102G2, CYP105, CYP107, CYP116, CYP155, CYP204, CYP293–298	6-Deoxyerythronolide B hydroxylase (CYP107A1 EryF) [35, 756]; erythromycin C-12 hydroxylase (CYP113A1 EryK) [757, 758]	E.g., 1JIO, 1Z8O (EryF), 2JJN, 3ZKP (EryK)

Table 6.1 (continued)

Organism	P450s	CYP families	Function(s)	PDB ID(s)
Sorangium cellulosum Soce56 (bacteria)	21	CYP109, CYP110, CYP117B1, CYP167A1, CYP210A1, CYP259–267	Fatty acid hydroxylase(s) (CYP109) [759, 760]; epoxidation of epothilones C and D (CYP167A1 EpoK) [152, 572, 761]; noriso-prenoid and sesquiterpene hydroxylase (CYP264B1) [762, 763]	1Q5D, 1Q5E (EpoK)
Sphingomonas paucimobi-lis (bacteria)	1	CYP152B1 ($P450SP_{\alpha}$)	Fatty acid α-hydroxylase [578, 764]	3AWM, 3VM4 ($P450SP_{\alpha}$)
Streptomyces avermitilis (bacteria)	33	CYP102, CYP105, CYP107, CYP125, CYP147, CYP154, CYP157–158, CYP170–171, CYP178–184	Fatty acid hydroxylase (CYP102D1) [692]); C1-(CYP105D6) [167] and C26-(CYP105P1) [167, 168] filipin hydroxylases; 1-deoxy-pentalenic acid hydroxylase (CYP105D7) [765]; 2-step allylic oxidation of epi-isozizaene to albaflavenone (CYP170A2) [205]; pentalenene hydroxylase (CYP183A) [550]	3ABB (CYP105D6), 3E5J (CYP105P1)
Streptomyces coelicolor A3(2) (bacteria)	18	CYP102B1, CYP105, CYP107, CYP154–159, CYP170A1	Fatty acid hydroxylase (CYP102B1) [766]); oxidase in coelibactin siderophore biosynthesis (CYP105N1) [169]; putative steroid oxi-dase with role in sporulation and antibiotic synthesis (CYP107U1) [145]; dipentaenone cyclization (CYP154A1) [111]; C–C coupling in flaviolin polymerization (CYP158A1 and CYP158A2) [195, 199]; 2-step allylic oxidation of epi-isozizaene to albaflavenone (CYP170A1) [203]	4FXB (CYP105N1), 1ODO (CYP154A1), 1GWI (CYP154C1) 2DKK (CYP158A1), 1S1F, (CYP158A2), 3DBG (CYP170A1)
Streptomyces scabiei (bacteria)	26	CYP102, CYP105, CYP107, CYP125, CYP145, CYP152, CYP154, CYP156–157, CYP179–180, CYP182, CYP246 (TxtC), CYP282–283, CYP1048A1 (TxtE)	Direct nitration of L-tryptophan with NO, O_2, redox partners, and NADPH (CYP1048A1 TxtE) [278, 279]; thaxtomin phenyl-alanyl di-hydroxylase (CYP246A1 TxtC, [275]) in thaxtomin A biosynthesis	4L36 (TxtE)
Sulfolobus acidocaldarius (archaea)	1	CYP119A1	Fatty acid hydroxylase and styrene epoxidase [91, 92, 98–100]	E.g., 1F4U, 1IO8, 1IO9 (CYP119A1)
Sulfolobus tokodaii 7 (archaea)	1	CYP119A2 (P450st)	Fatty acid hydroxylase and styrene epoxidase [95, 97, 100]	1EU8, 3B4X (CYP119A2)
Thermus thermophilus (bacteria)	1	CYP175A1	β-Carotene [767], zeaxanthin [768] and monoenoic fatty acid [102] hydroxylase	1N97, 1WIY (CYP175A1)

PDB Protein Data Bank

6.1.2 Microbial P450 Classification and Sequence Conservation

The current system for managing and annotating P450 enzymes in the P450 enzyme superfamily (developed by David Nelson) [18–20] places the P450s in the same family if they share 40% or more identity at the amino acid level. P450s with lower identity are classified into different families (CYP1, CYP2, etc.), while those sharing 55% or more amino acid sequence identity define subfamilies within a P450 family, denoted by a capital letter (CYP1A, etc). Individual members of a subfamily then receive consecutive numbers (CYP1A1, 1A2, etc.). The prokaryotic (bacterial and archaeal) P450s are currently classified within families CYP101–CYP299 and CYP1001–CYP1050, while the yeast/fungal P450s are placed in families from CYP52 to CYP69, and also in various families from CYP501 upwards [18]. The CYP51 family contains sterol 14α demethylase P450s from almost all eukaryotes and from a small number of bacteria—including CYP51B1 from the human pathogen *Mycobacterium tuberculosis* [21, 22]. Thus, the CYP51s are the only P450 family found from prokaryotes through to man, leading to the suggestion that CYP51 is a progenitor P450 in the enzyme superfamily [23]. However, as discussed further below, the absence of the *CYP51* gene from some eukaryotes and the complexity of the CYP51 reaction suggests that it itself may have evolved from another primordial P450 function.

Microbial P450s have diverse catalytic functions (with numerous isoforms remaining uncharacterized) and divergent sequences, but adopt the same general structural fold as their eukaryotic counterparts (despite the absence of an N-terminal transmembrane segment in the bacterial/archaeal forms compared to eukaryotic P450s; Fig. 6.1). However, the advent of genome sequencing and the ever-increasing numbers of P450 sequences emerging from such analyses have thrown up several exceptions to the 'traditional' model of a P450 with a number of implicitly conserved amino acids residues and retained motifs (e.g., the characteristic 'heme binding' motif FXXGXXXCXG (where X is any amino acid) around the cysteine axial ligand to the heme iron, the protonating threonine (or serine) of the I-helix that interacts with the iron-bound dioxygen, and the K-helix EXXR motif (with roles in protein folding and heme insertion) [24]. The amino acid sequences of members of the P450 superfamily have evolved on such a scale that there is no longer any completely conserved amino acid residue or invariant region common to all P450 members [25, 26]. The profound influence of mutations on P450 properties is evident from the fact that even a single amino acid change can alter substrate specificity or have other dramatic effects on catalysis [27–29]. The I-helix threonine/serine (Thr252 in the well-characterized P450cam) has roles in protonation of the ferric-superoxo and ferric-hydroperoxo P450 catalytic cycle intermediates, leading to efficient O–O bond scission during molecular oxygen activation [24, 30–32]. This residue was thought to be implicitly conserved in all P450s, with mutagenesis studies showing a loss of activity and catalytic uncoupling upon substitution of the threonine residue in key P450 enzymes [31, 33]. The *Saccharopolyspora erythraea* CYP107A1

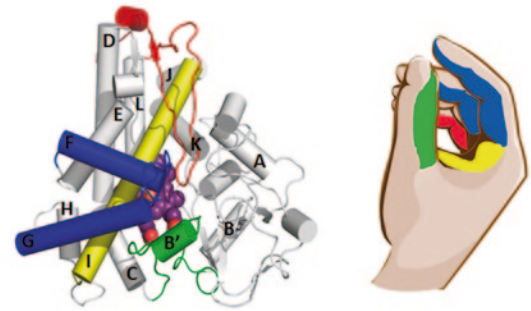

Fig. 6.1 Overview of the general P450-fold. The *left* panel shows a cartoon representation of a typical P450 structure (P450cam; PDB 2CPP), with helices labeled according to existing conventions [12, 24]. The heme cofactor is shown in atom colored spheres. Key structural elements involved in substrate binding are shown in color: the BC loop (in certain cases containing the B′ helix) in *green,* the FG helices in *blue,* the central I-helix in *yellow,* and the C-terminal region in *red*. The *right* panel shows a cartoon representation of a general model for P450 substrate binding: the key structural elements defined in the *left* panel act as the fingers of the hand (which can grab an object, in this case the substrate), with the I-helix and heme resembling the palm. *PDB* Protein Data Bank

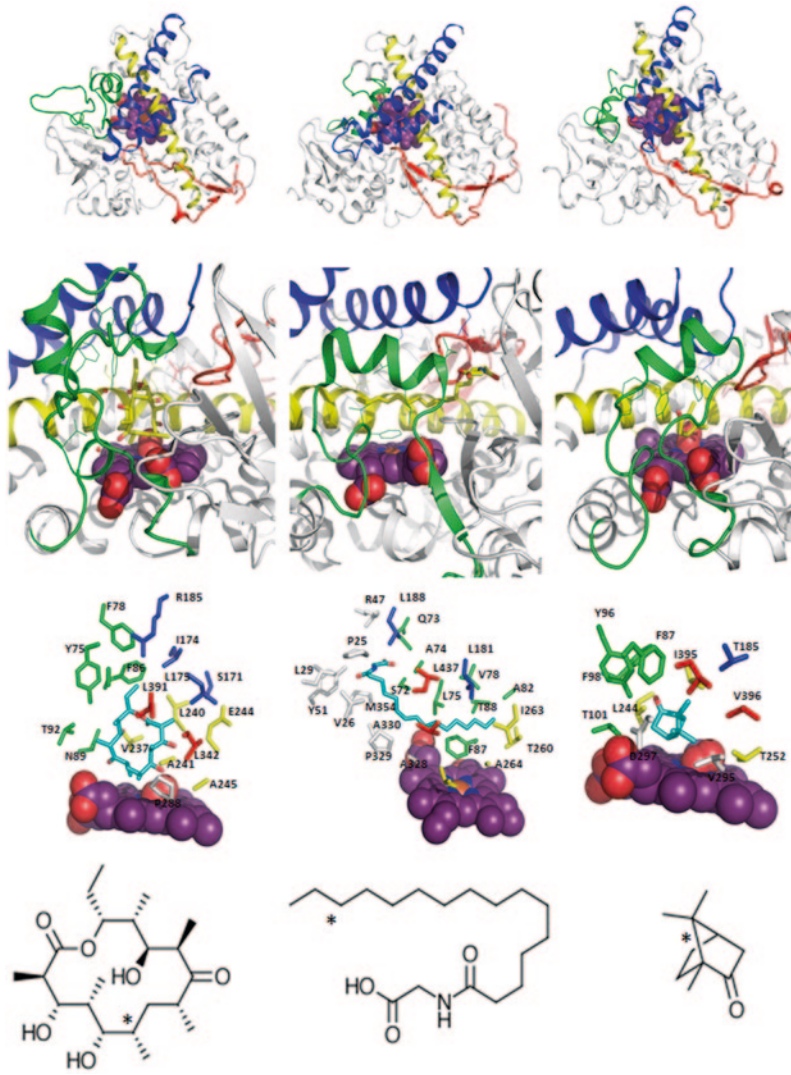

Fig. 6.2 A detailed comparison of three model microbial P450 enzymes. The *left* column depicts P450 EryF (PDB 1OXA) [34], the *middle* column the P450 BM3 heme domain (PDB 1JPZ) [771], and the *right* column P450cam (PDB 2CPP) [12]. The first row shows the general fold of each P450, color-coded as in Fig. 6.1. The second row shows a detailed view of the active site region, with the bound substrate shown in sticks. The third row shows the key residues involved in substrate binding, represented in sticks and colored according to structural elements as defined in Fig. 6.1. The substrate is shown with cyan carbon atoms. Where available, the structure of the ferrous–oxy complex is shown, with the heme and bound dioxygen in space filling sphere representation. The last row shows the structures of the respective substrates, with an *asterisk* defining the (main) position of oxidation of the substrate

(EryF), a 6-deoxyerythronolide B (6-DEB) hydroxylase (Fig. 6.2) involved in erythromycin biosynthesis, was the first example of a P450 where the 'conserved' threonine was found to be absent, with an alanine replacement at the relevant position in the P450 scaffold (Ala245). The alanine lacks a functional group able to protonate iron-bound dioxygen. Instead, the CYP107A1 6-DEB substrate provides a hydroxyl group in the C5 position that plays a role similar to the threonine in so-called substrate assisted catalysis, and thus confers specificity to the 6-DEB sub-

strate [34–36]. Mutagenic substitution of the CYP107A1 alanine residue to a threonine (A245T) restores the conventional dioxygen protonating role of the threonine, enabling CYP107A1 to oxidize a variety of substrates related to 6-DEB, but lacking the hydroxyl moiety, and thus highlighting the importance of this threonine/serine residue for efficient oxygen activation in most P450s [37]. There are now multiple examples of P450s that lack the I-helix threonine. For example, CYP176A1 (P450cin) from *Citrobacter braakii* involved in the hydroxylation of 1,8-cineole to produce 6-β-hydroxycineole, which has an asparagine (Asn242) residue instead of the threonine [38]. This residue was found to be involved in the regio- and stereo-selective oxidation of cineole, forming a hydrogen bond with the substrate oxygen, and not directly replacing the role of the conserved threonine in oxygen activation itself. Thus, the source of a proton donor for dioxygen activation is still unresolved in CYP176A1 [39, 40]. The EXXR motif in the P450 K-helix, thought to be crucial for P450 tertiary structure and for heme binding by forming a set of salt bridge and hydrogen-bonding interactions, was also considered to be invariant, with substitutions of the glutamic acid or arginine residue(s) having severely detrimental effects on P450 structural integrity and enzyme activity (e.g., [41]). However, CYP156B1 and the CYP157 family P450s appear to be exceptions to the rule, with this motif absent in *Streptomyces coelicolor* CYP157C1-4 [42, 43] and also from other emerging members of the CYP157 family, mainly in *Streptomyces* spp. [19, 44].

The ancestral 'progenitor' of the cytochrome P450 superfamily is still unclear, with several hypotheses put forward. These include a possible anaerobic reductase role for the original P450, similar to that described for fungal nitric oxide reductases (CYP55A family) [45, 46], or a peroxygenase-type role, as described for plant allene oxide synthases (AOS, CYP74A family) and hydroperoxide lyases (CYP74B family) [47] prior to P450 acquisition of oxygen-binding/activation capabilities. It has also been postulated that P450 oxygen-binding capacity may have evolved to enable the detoxification of molecular oxygen, and to enable survival of organisms as oxygen levels began to rise in the environment as a result of cyanobacterial photosynthesis [25, 48]. Lipids and sterols, found in geological samples, have been around since the Precambrian period and were suggested to be involved in the early metabolic roles of P450s [49, 50]. Oxygenated lipids and sterols, the products of early detoxification events, could hold important roles in the evolution of vesicles, membranes and cellular life, and thus act as signaling molecules, possibly pointing to the probable early roles of ancient P450s. It thus appears that the evolution of P450s may be related to both atmospheric and geological events dating back 2 billion years [25, 46, 51]. It was originally speculated that CYP51 might be the ancestral P450, due to the availability of ancient sterols and its ability to produce 14α-demethylated sterols (although rarely seen in bacteria), and as a result of its presence in all of the different domains of life. It was further considered that all eukaryotic P450s may have derived from a single CYP51 [49, 52, 53]. However, this hypothesis is now considered unlikely, due in part to the fact that the sterol 14α-demethylation (the specific sterol substrates showing some variation depending on the source of the particular CYP51 enzyme) requires three successive oxidative events, culminating in a carbon–carbon bond scission and the release of formic acid. This relatively complex CYP51 reaction mechanism is likely derived from a 'simpler' P450-mediated oxidation process and is sufficiently specialized that it is not likely to be the ancestral P450 trait [25, 54, 55]. Furthermore, there are also arguments for lateral gene transfer of plant CYP51s to bacteria [56]. The absence of *CYP51* genes in the archaea and in the majority of bacteria, as well as in some eukaryotes (e.g., certain insects and nematodes), suggests that although CYP51 is likely an early P450, it is not the progenitor in the P450 gene superfamily. However, the presence of P450s in archaea, bacteria, and eukaryotes suggest that the most primitive P450 may have emerged early in the evolution of life forms.

The following sections illustrate the diversity of P450 systems in the microbial world, with key examples of crucial chemical reactions

performed, illustrations of the structures and mechanisms of important microbial P450s, and examination of novel redox and nonredox partner protein interactions in microbial P450 enzymes.

6.2 Microbial Diversity of P450s

6.2.1 The Extent of P450s in Microbial Genomes

The numbers of P450 (*CYP*) genes in microbial organisms differ extensively (Table 6.1), even between species of the same genus. Genome sequencing projects continue to reveal genes for new P450s and for novel classes of these enzymes from the Bacteria and Archaea domains, and for the fungal kingdom of the Eukarya domain. However, archaea and bacteria generally contain relatively few P450s (in comparison to most eukaryotes) with certain organisms having no *CYP* genes present (e.g., *Escherichia coli* [57] and *Helicobacter pylori* [58, 59]). Moderate P450 numbers (2–8 P450s/genome) and extents of genetic diversity are observed in *Bacillus* species genomes across a limited number of *CYP* gene families (*CYP102, CYP106, CYP107, CYP109, CYP134, CYP152,* and *CYP197,* with 51 genes identified at time of preparation of this chapter) [19, 59–61]. Several of the *Bacillus* P450s have undefined or uncertain physiological roles, although numerous studies have been done on certain *Bacillus* spp. P450s, including intensive characterization of the CYP102A1 (P450 BM3) P450-cytochrome P450 reductase (CPR) fusion enzyme (see the section 'Microbial P450-(redox) partner fusion enzymes'). P450 BM3 is a highly efficient fatty acid monooxygenase found in a number of *Bacillus* spp. as well as in other bacteria, but one for which a definitive physiological function remains elusive to date [28]. Other characterized *Bacillus* P450s include isoforms that possess fatty acid or steroid hydroxylating activities [60, 62–64]. In contrast to *Bacillus* and many other bacteria, actinobacteria often contain large numbers of P450 genes, e.g., in mycobacterial spp. For example, there are 17, 20, 21, 39, and 47 CYP genes in *Mycobacterium bovis AF2122/97, M. tuberculosis* H37Rv, *M. ulcerans Agy99, M. smegmatis MC2155,* and *M. marinum,* respectively [59]. As is the case for many other microbes (and also for several higher eukaryotes), P450 protein expression and functional characterization has not kept pace with data emanating from genome sequencing projects, and thus most mycobacterial P450s have unknown roles. However, for mycobacterial P450s where biochemical data are available, a variety of unusual catalytic functions have been identified including roles in bacterial virulence and novel secondary metabolite production [65, 66] (see the section 'Mycobacterial P450s'). Other actinobacteria, such as *Streptomyces* spp., are often also rich in P450s, with roles commonly in antibiotic biosynthesis or in the production or other natural products. For instance, there are 33 *CYP* genes in *Streptomyces avermitilis MA-4680,* 65 in *S. clavuligerus,* 18 in *S. coelicolor A3 (2)* and *S. lividans,* 21 in *S. venezuelae,* and 27 in *S. griseus* [59, 67, 68]. The majority of the actinobacterial P450s are unique to their own genus, although there are also a proportion (particularly from soil dwelling organisms) that encompass the CYP105 and CYP107 families, and which are also found in other bacteria [19]. Not all actinobacteria contain a large P450 complement, important examples being the leprosy causing *M. leprae* [69] and the gastrointestinal, micro-aerotolerant *Bifidobacterium longum,* which is considered to have probiotic properties through its production of lactic acid [70]. These bacteria have one P450 each, further illustrating the large differences in numbers of P450s in different microbial organisms. The gliding myxobacteria have complex life cycles and are of pharmaceutical and physiological interest, producing a wide array of secondary metabolites with useful activities including anticancer therapeutics (e.g., epothilones), as well as antibacterial and antiviral agents [71]. Myxobacteria also typically contain a large pool of P450s, with good examples being *Sorangium cellulosum Soce56* with 21 P450s identified, *Stigmatella aurantiaca DW4/3–1* (18 P450s), *Haliangium ochraceum DSM 14365* (17 P450s), and *Myxococcus xanthus DK1622* (7 P450s). Among the myxobacterial P450 genes identified, there are members

of the CYP109, CYP110, CYP117, and CYP124 families, as well as new myxobacteria-specific P450 families [72].

Moving into the eukaryotic microbes, the filamentous fungus *Aspergillus nidulans* has a larger P450 complement than all other lower organisms known to date, with 111 P450 genes identified across 89 families [15]. Some of the largest microbial P450 numbers occur in white and brown rot fungi, where the P450s have important roles in the breakdown of plant material. The model white rot fungus *Phanerochaete chrysosporium* is involved in the biodegradation of lignin and the metabolism of polycyclic hydrocarbons and other xenobiotics [73]. The *P. chrysosporium* genome sequence revealed the presence of 149 *CYP* genes, with some having direct roles in lignin breakdown, and displaying variable gene expression levels under a variety of environmental conditions. These P450 enzymes (and other redox enzymes, including peroxidases) are of obvious biotechnological interest with potential roles in biodegradation of plant biomass and generation of chemicals and biofuels [74–77]. The P450 repertoire appears to be just as large in other white rot species, e.g., *Bjerkandera adusta* with 199 *CYP* genes, and *Ganoderma* sp. and *Phlebia brevispora* each with 209 P450s, although characterization of the vast majority of these enzymes remains to be done [78–82]. The genome of the brown rot fungus *Postia placenta* identified an even greater P450 complement with 250 genes across 41 P450 families present, with 184 of these shown to have activity with stilbene and a number of other compounds [77, 83]. However, the molecular mechanisms of biodegradation by brown rot fungi are not nearly as intensively studied or as well understood as those for their white rot fungal counterparts.

6.2.2 P450s in the Archaea

Evidence of P450s in the archaea suggests an early evolution for these proteins. Archaeons are among the earliest forms of life. It is now generally believed that the archaea and bacteria developed separately from a common ancestor nearly 4 billion years ago [84]. Millions of years later, the ancestors of today's eukaryotes split off from the archaea, suggesting closer relations between eukaryotes and archaea than between archaea and the prokaryotes/bacteria. In contrast to eukaryotes (where P450s are almost invariably present), archaea, like most eubacteria, do not contain large numbers of P450s. However, recent genome sequencing projects have revealed a limited number of P450s in many archaeons [19, 85]. There are three main phyla of archaea: (i) the Crenarchaeota, which are characterized by their ability to tolerate extremes in temperature and acidity; (ii) the Euryarchaeota, which include methanogens and halobacteria; and (iii) the Korarchaeota (or xenarcheota), which are found in high-temperature hydrothermal systems [84, 86, 87]. Among these, three main types of archaea are some subtypes, which include methanogens (producing methane as a metabolic by-product), halophiles (requiring high NaCl concentrations for survival), thermophiles, and psychrophiles (which grow at unusually high and low temperatures, respectively).

The first P450 identified in an archaeon was discovered fortuitously by Wright et al. during studies to clone thymidylate synthase from the acidothermophilic archaeon *Sulfolobus acidocaldarius* (previously named *Sulfolobus solfataricus*), who identified a P450-like gene sequence containing a consensus P450 heme-binding motif [88]. This enzyme was classified as CYP119, and subsequently as CYP119A1 [89, 90]. To date, there are four crystal structures of thermophilic P450s published. Two of these are for CYP119 family enzymes: CYP119A1 from *Sulfolobus acidocaldarius* (e.g., PDB 1F4T) [91, 92] and CYP119A2 from *Sulfolobus tokodaii* strain 7 (P450st, PDB 1UE8) [93] (Fig. 6.3). In efforts to provide a redox partner system for CYP119A1, the P450cam redox partners putidaredoxin reductase/putidaredoxin (PDR/PD) and spinach ferredoxin reductase/ferredoxin (FDR/FD) proved to be inefficient electron donors [94]. However, it was subsequently demonstrated that CYP119A1 can obtain electrons from a *S. tokodaii* 2-oxoacid: ferredoxin oxidoreductase and FD system, with a 2-oxoacid such as pyruvate as the electron

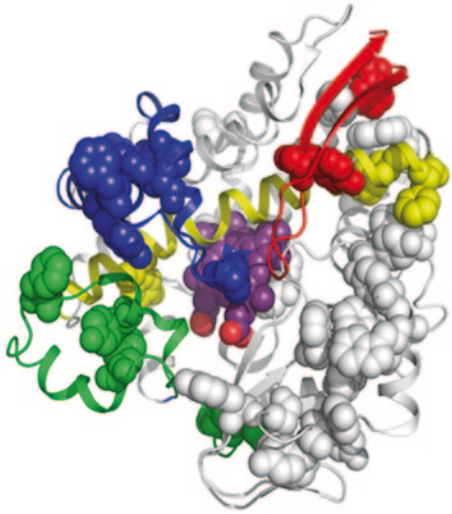

Fig. 6.3 The structural basis of thermostability in CYP119. An unusually large network of aromatic residues (here shown in space filling spheres, colored by structural element) has been suggested to contribute to thermostability of *S. solfataricus* CYP119A1 (PDB 1F4U) [92]

donor [95]. Cloning and expression/purification of a homologous thermostable 2Fe–2S FD and pyruvate-dependent ferredoxin oxidoreductase led to the reconstitution of lauric acid hydroxylase activity with CYP119A1, and thus to the description of the first example of a non-NAD(P)H-dependent reductase partner system for a P450 enzyme [96]. More recently, CYP119A2 was shown to be reduced directly by nicotinamide adenine dinucleotide phosphate (NAD(P)H) and to catalyze epoxidation of styrene, suggesting a novel route to oxidation chemistry in this P450 that bypasses redox partner proteins [97] (see the section 'P450s from thermophilic microbes and novel redox systems for Sulfolobus P450s' in *Redox partner systems and their diversity in microbes*). The endogenous substrates of the archaeal CYP119A enzymes are still unknown, although they do possess fatty acid monooxygenase activity, showing hydroxylation of lauric acid and the binding of saturated C12–C18 fatty acids [98, 99]. CYP119A1 also catalyzes the H_2O_2-dependent epoxidation of styrene and of *cis*- and *trans*-β-methylstyrenes, albeit with lower affinities and catalytic rates than observed with fatty acids [94]. The structures of CYP119A1 and CYP119A2 show a high degree of similarity and contain two large aromatic clusters that are not present in mesophilic P450s [91–93] (Fig. 6.3). These clusters are likely to contribute to their thermostability, along with potential stabilization from a higher density of salt bridges, and from a lower density of alanine residues coupled to a higher density of isoleucine residues, which is thought to contribute to better side-chain packing [92, 100].

CYP175A1 from the Gram-negative thermophilic eubacterium *Thermus thermophilus* has also been crystallized and structurally characterized (PDB code 1N97) [101], and was reported to be a β-carotene hydroxylase in production of the carotenoid zeaxanthin [101] and to possess monoenoic fatty acid oxygenase activity [102]. In contrast to the CYP119 P450s, CYP175A1 does not possess large aromatic amino acid clusters and its thermostability is instead proposed to be related to salt-bridge networks whereby charged residues are assembled to form multiple salt linkages rather than individual electrostatic interactions [100, 101]. CYP231A2 comes from the thermoacidophilic euryarchaeon *Picrophilus torridus* PTO1399 (PDB code 2RFB), the most acidophilic organism known that thrives optimally at 60 °C and pH 0.7, but can grow even at pH 0 [103]. CYP231A2 has no known function as yet, but is the smallest structurally characterized P450 (39.56 kDa and 352 residues), and its small size, together with factors such as low surface-to-volume ratio due to short loops and dispensing with excess secondary structure, may be major factors in its thermostability [103]. However, its T_m is only 65 °C compared to 95 °C for CYP119A1. A further P450 is found in *Picrophilus torridus* PTO0085 (CYP232A2; 44.36 kDa), and progesterone hydroxylase activity in the thermophilic Gram-positive *Geobacillus thermoglucosidasius* strain 12060 and *Geobacillus stearothermophilus* (both formerly classified in the genus *Bacillus*) has also been reported [104, 105]. While the study of the structure and function of P450 enzymes from thermophilic archaeons (and from thermotolerant bacteria) is in its infancy, the relative ease with which proteins from these organisms can be crystallized for

structural determination is one obvious reason for their continued study, which should provide novel information on mechanisms of P450 protein thermostability. The ability to interrogate the catalytic processes of P450s and (in particular) the nature of transient iron–oxo species in the catalytic cycle is another attractive advantage for studying thermostable P450 enzymes at ambient (or lower) temperatures. Indeed, the major breakthrough made by Rittle and Green in trapping and definitively characterizing the reactive compound I (ferryl–oxo porphyrin radical cation) species (Fig. 6.4) in P450 was achieved using the *S. acidocaldarius* CYP119A1 (see the section 'P450s from thermophilic microbes and novel redox systems for Sulfolobus P450s' in *Redox partner systems and their diversity in microbes*) [13].

Thus, the majority of the biodiverse and rapidly numerically increasing microbial P450s have only been identified and characterized at the gene/amino acid sequence level. On the basis of recent studies that have revealed several unexpected functions for microbial P450s (e.g., cyclodipeptide oxidation, microbial toxin, and dinitrogen oxide (N_2O) synthesis [65, 106, 107]), the expectation is that the characterization of many of these 'orphan' microbial P450s will reveal an even greater range of functional capabilities in the P450 superfamily. At present, P450 protein sequence analysis alone is usually inadequate for identification of substrate specificity (unless the sequence is highly related to another P450 of established substrate selectivity). With even small changes in amino acid sequence in key regions being sufficient to cause major alterations to substrate recognition and/or position of substrate oxidation, the assignment of catalytic functions to the growing number of orphan microbial P450s (including representatives from unique families) is challenging. Much work lies ahead in order to gain knowledge of physiological roles and the chemical mechanisms of numerous such P450 enzymes. Such work will undoubtedly require novel approaches, and embrace technologies such as gene knockout/knockdown coupled to analysis of the metabolome, and activity screening using diverse compound libraries [108–111].

6.2.3 P450s in Bacteria

There are more than 1300 bacterial P450 sequences within 350 distinct families which encompass the family numbers from CYP101 to CYP299 and from CYP1001 to CYP1050, along with bacterial members of the CYP51 clan [19]. Bacterial P450s were originally considered as mainly being involved in the catabolism of exogenous compounds to allow their utilization as a source of nutrition. Compared to various eukaryotic (particularly mammalian hepatic) counterparts, it is the case that several bacterial P450s are more 'specialist', with rather restricted substrate specificity ranges. Indeed, in many cases, the natural substrates of bacterial P450s are unknown, with numerous 'orphan' bacterial *CYP* genes identified from ongoing genome sequencing projects. For most of the membrane-bound P450s, structural changes including movement of the smaller *beta* domain towards the proximal side of the heme plane may be important for increasing active site size and enabling substrate/product entry/exit from the membrane. However, structures of bacterial P450s, in general, do not show the same plasticity and flexibility as observed for various eukaryotic P450s [112] (Fig. 6.2). The prokaryotic enzymes lack the N-terminal membrane anchor regions found in eukaryotic P450s, and are thus soluble, cytoplasmic enzymes (rather than being associated with microsomal or adrenal mitochondrial membranes). This has helped facilitate the studies of their structural and biochemical properties, particularly with respect to being able to express and purify sufficient amounts of soluble P450 to enable studies using protein crystallography and spectroscopic methods [12]. The P450 representatives in the PDB at present are mainly prokaryotic P450 structures, due to their superior stability and relative ease in handling compared to the membrane-bound P450 enzymes (Table 6.2). In contrast to microsomal P450s, which use CPR or CPR/cytochrome b_5 as electron donating redox partners for catalysis, bacterial P450s can use a more diverse mixture of redox partner enzymes. Indeed, the list of prokaryotic redox partners has increased steadily in recent years (see the section 'Redox partners

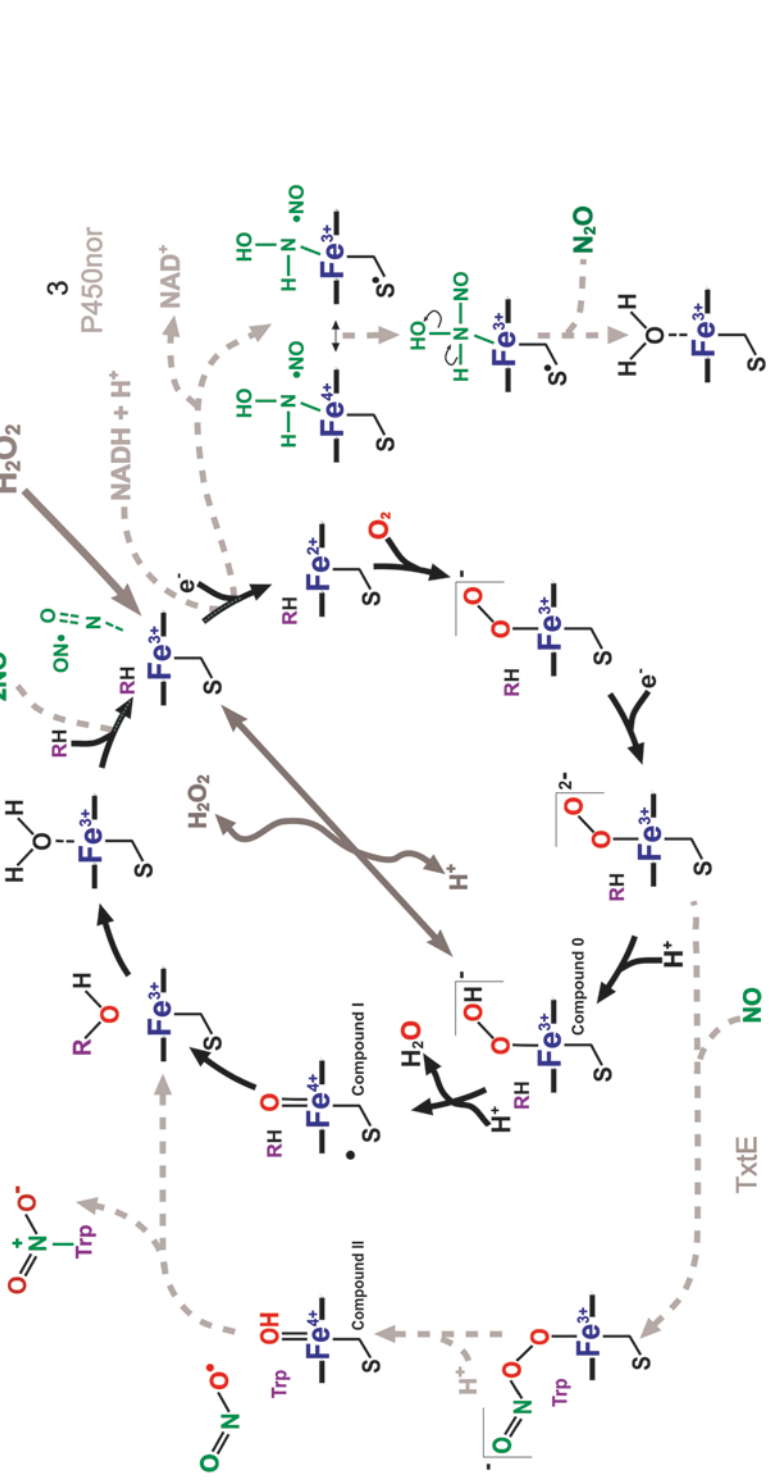

Fig. 6.4 An expanded P450 catalytic cycle. The scheme shows: (*1*) The conventional catalytic cycle with R as substrate (e.g., [285]). (*2*) The peroxide shunt pathway that bypasses the need for external electrons, converting the substrate-bound ferric P450 to the reactive compound I (via compound 0), as also described for the peroxygenase CYP152 family enzymes [574, 575, 577]. (*3*) The reaction of P450nor (CYP55A1) that binds two molecules of nitric oxide (*NO*) and receives electrons directly from NADH without the need of accessory redox partner proteins to generate dinitrogen oxide (N_2O) [847]. (*4*) The proposed mechanism of TxtE (CYP1048A1) that utilizes NO and O_2 to catalyze the direct nitration of tryptophan in the biosynthesis of the toxin thaxtomin [107]

Table 6.2 Microbial P450 structures. The structures of microbial P450s in chronological order from PDB deposition, highlighting key features. Color coding is as for Table 6.1 with light gray for archaea and mid-gray for fungi

P450	Organism	PDB codes	Ligands	Function	Refs
CYP101A1 (P450 cam)	*Pseudomonas putida*	E.g., 2CPP, 1AKD, 4JWU	E.g., Camphor, CO, thiocamphor, adamantine	Camphor 5-exo-hydroxylase	E.g., [12, 769, 770]
CYP102A1 (P450 BM3)	*Bacillus megaterium*	E.g., 1FAG, 2HPD	Palmitoleic acid, N-palmitoyl glycine, DMSO	Fatty acid, e.g., arachidonic monooxygenase	E.g., [271, 701, 771, 772]
CYP108A1 (P450 terp)	*Pseudomonas* sp.	1CPT		α-Terpineol hydroxylase	[120]
CYP107A1 (P450 EryF)	*Saccharopolyspora erythraea*	E.g., 1OXA, 1Z8O, 1EGY, 1EUP	E.g., 6-deoxyerythronolide B, androstenedione, 9-aminophenanthrene, ketoconazole	6-Deoxyerythronolide B hydroxylase in erythromycin biosynthesis	E.g., [34, 36, 773]
CYP55A1 (P450 nor)	*Fusarium oxysporum*	1ROM	E.g., NAAD, NO, CO, N-butyl isocyanide	Nitric oxide reductase	E.g., [327, 328, 774, 775]
CYP119A1	*Sulfolobus solfataricus*	1F4T, 1F4U, 1IO9, 1IO7	4-Phenylimidazole	Fatty acid (e.g., lauric acid) hydroxylase, styrene epoxidase	[91, 92]
CYP51B1 (MtbCYP51)	*Mycobacterium tuberculosis*	1EA1, 1EPX, 1H5Z	E.g., 4-phenylimidazole, fluconazole, estriol, 4,4′-dihydroxybenzophenone	Sterol (e.g., obtusifoliol) 14α-demethylase	E.g., [408, 437, 438]
CYP165B3 (OxyB)	*Amycolatopsis orientalis*	1LFK, 1LG9, 1LGF		Oxidative phenol coupling of CD-rings in vancomycin biosynthesis	[248]
CYP154C1	*Streptomyces coelicolor* A3(2)	1GWI		12- and 14-carbon macrolactone monooxygenase E.g., narbomycin hydroxylase	[188]
CYP121A1	*Mycobacterium tuberculosis*	E.g., 1N4O, 2IJ7, 3G5H, 4G2G, 4ICT	E.g., Fluconazole, cYY, 4,4′-(1H-1,2,3-triazole-1,5-diyl) diphenol, cYF	C–C bond formation of cyclodityrosine (cYY) to form mycocyclosin	E.g., [65, 187, 414, 415, 420]
CYP175A1	*Thermus thermophilus*	1N97		β-Carotene, zeaxanthin, and monoenoic fatty acid hydroxylase	[101]
CYP152A1 (P450 Bsβ)	*Bacillus subtilis*	1IZO, 2ZQJ, 2ZQX	Palmitoleic acid	Fatty acid (e.g., myristic acid) hydroxylase. H_2O_2-dependent peroxygenase	[577, 776]
CYP167A1 (P450 EpoK)	*Sorangium cellulosum*	1Q5D, 1Q5E, 1PKF	Epothilone B, epothilone D	Epothilone C and D epoxidation	[761]
CYP165C4 (OxyC)	*Amycolatopsis orientalis*	1UED		Biaryl cyclization of AB-rings in vancomycin biosynthesis	[249]

Table 6.2 (continued)

P450	Organism	PDB codes	Ligands	Function	Refs
CYP154A1	*Streptomyces coelicolor* A3(2)	1ODO		Possibly involved in polyketide metabolism	[180]
CYP176A1 (P450cin)	*Citrobacter braakii*	E.g., 4FB2, 1T2B, 4FYZ	1,8-Cineole, NO	1,8-Cineole monooxygenase	E.g., [121, 777]
CYP119A2 (P450st)	*Sulfolobus tokodaii*	1UE8		Fatty acid (e.g., lauric acid) hydroxylase, styrene epoxidase, direct electron transfer from NADH	[93, 97]
CYP158A2	*Streptomyces coelicolor* A3(2)	E.g., 1SE6, 1T93, 1S1F, 2D0E	Flaviolin, 4-phenylimidazole, 2-hydroxynaphthoquinone	Oxidative phenolic coupling in flaviolin polymerization	E.g., [195, 197, 198]
CYP107L1 (PikC)	*Streptomyces venezuelae*	E.g., 2BVJ, 2CD8, 2C7X	Narbomycin, YC-17	12- and 14-Carbon macrolactone (e.g., narbomycin and YC-17) hydroxylase in pikromycin synthesis	E.g., [208, 210, 211]
CYP199A2	*Rhodopseudomonas palustris* CGA009	2FR7, 4DNJ	4-Methoxybenzoic acid	Oxidation of para-substituted benzoic acids (83% identity to CYP199A4)	[778, 779]
CYP158A1	*Streptomyces coelicolor* A3(2)	2NZA, 2NZ5, 2DKK	Naphthalene-1,2,4,5,7-pentol, imidazole	Oxidative phenolic coupling involved in flaviolin polymerization	[195]
CYP245A1 (StaP)	*Streptomyces* sp. tp-a0274	2Z3T, 2Z3U, 3A1L	Chromopyrrolic acid, 11,11′-dichlorochromopyrrolic acid	Aryl–aryl coupling of chromopyrrolic acid in staurosporine biosynthesis	[154, 272]
CYP105AB3 (P450 MoxA)	*Nonomuraea recticatena*	2Z36		Nonspecific, e.g., compactin, luciferase and oleanolic acid hydroxylation in xenobiotic degradation and natural product synthesis	[780]
CYP130A1	*Mycobacterium tuberculosis*	E.g., 2UUQ, 2UVN, 2WHF	Econazole, 1-(3-methylphenyl)-1H-benzimidazol-5-amine	Substrate/function unknown	[108, 409]
CYP231A2	*Picrophilus torridus* PTO1399	2RFB, 2RFC	4-Phenylimidazole	Substrate/function unknown	[103]
CYP105A1 (P450 SU-1)	*Streptomyces grisoleus*	E.g., 3CV9, 2ZBZ	1,25-Dihydroxyvitamin D3	Vitamin D3 1α and 25-hydroxylation (conversion to active form)	[175, 176]
CYP120A1	*Synechosystis* sp.	2VE3, 2VE4	Retinoic acid	Retinoic acid hydroxylase	[781]
CYP248A1 (CalO2)	*Micromonospora echinospora* (or *purpurea*)	3BUJ		Orsellinic acid hydroxylase in calicheamicin biosynthesis	[230]
CYP107H1 (BioI)	*Bacillus subtilis*	3EJB, 3EJD, 3EJE	Hexadec-9Z-enoic acid-(ACP), octadec-9Z-enoic acid-(ACP)	Oxidative cleavage of ACP-bound fatty acids in pimelic acid biosynthesis	[131]

Table 6.2 (continued)

P450	Organism	PDB codes	Ligands	Function	Refs
CYP105P1	*Streptomyces avermitilis*	3E5J, 3E5K, 3ABA	4-Phenylimidazole, filipin I	C26-Filipin hydroxylase	[167, 168]
CYP170A1	*Streptomyces coelicolor* A3(2)	3DBG, 3EL3	Epi-isozizaene	2-Step allylic oxidations of epi-isozizaene to albaflavenol(s) in albaflavenone biosynthesis	[203]
CYP113A1 (EryK)	*Saccharopolyspora erythraea*	2JJN, 2JJP, 2JJO	Erythromycin D, ketoconazole, clotrimazole	Erythromycin D (C12)-hydroxylase, in erythromycin A biosynthesis	[757, 782]
CYP177A1 ($XplA_{HD}$)	*Rhodococcus* sp. str11Y	2WIV, 2WIY, 4EP6	Imidazole	Reductive denitration of hexa-hydro-1,3,5-trinitro-1,3,5-triazine (royal demolition explosive, RDX)	[135, 137]
CYP124A1	*Mycobacterium tuberculosis*	2WM5, 2WM4	Phytanic acid	ω-hydroxylation of methyl-branched lipids, cholesterol C26 hydroxylation	[395]
CYP125A1	*Mycobacterium tuberculosis*	E.g., 3IVY, 3IW1, 3IW2, 2X5W	Androstenedione, econazole, cholest-4-en-3-one	Cholesterol and cholest-4-en-3-one 26-oxidase	[360, 363]
CYP105D6	*Streptomyces avermitilis*	3ABB		C1-Hydroxylation of filipin	[167]
CYP146A1 (OxyD)	*Amycolatopsis mediterranei*	3MGX		B-tyrosine hydroxylation in vanco-mycin biosynthesis	[237]
CYP101D1	*Novosphingobium aromaticivorans*	E.g., 3LXH, 4C9K, 4C9L	Camphor, 5-exo-hydroxycamphor, CN	Camphor 5-exo hydroxylase	[118, 783]
CYP107BR1 (P450vdh)	*Pseudonocardia autotrophica*	3A4G, 3A5O	Vitamin D3	Vitamin D hydroxylase—conversion to active form	[784]
CYP161A2 (PimD)	*Streptomyces natalensis*	2X9P, 2XBK	4,5-Desepoxypimaricin	4,5-Desepoxypimaricin epoxidase in pimaricin biosynthesis	[216]
CYP134A1 (CypX)	*Bacillus subtilis*	3NC3, 3NC5, 3NC6, 3NC7	1-Phenylimidazole, 2-phenylimidazole	Cyclo-L-Leucyl-L-Leucyl (cLL) oxidation to pulcherriminic acid	[393]
CYP142A1	*Mycobacterium tuberculosis*	2XKR		Cholesterol and cholest-4-en-3-one 26 oxidase	[362]
CYP101D2	*Novosphingobium aromaticivorans*	3NV5	Camphor	Camphor 5-exo hydroxylase	[119]
CYP165D3 (OxyE)	*Actinoplanes teichomyceticus*	3O1A, 3OO3		Oxidative phenol coupling of FG-rings in teicoplanin biosynthesis	[239, 240]
CYP151A (AurH)	*Streptomyces thiolteus*	3P3L, 3P3X, 3P3O, 3P3Z	Ancymidol	Oxidation and ring formation to con-vert deoxyaureothin to aureothin	[226]

Table 6.2 (continued)

P450	Organism	PDB codes	Ligands	Function	Refs
CYP101C1	*Novosphingobium aromaticivorans*	3OFT, 3OFU	Hexane-2,5-diol, β-ionone	Ionone derivative (e.g., α- and β-ionone and β-damascone) hydroxylation	[750]
CYP152B1 (P450SPα)	*Sphingomonas paucimobilis*	3AWM, 3AWP, 3WAQ	Palmitic acid	Fatty acid α-hydroxylase. (H_2O_2-dependent peroxygenase)	[578]
CYP107E1 (MycG)	*Micromonospora griseorubida*	E.g., 2YGX, 2YCA, 2Y98, 2Y5Z	Mycinamicin III, IV and V	C14-Hydroxylation and C12/C13-epoxidation on macrolactone ring of mycinamicin	[311]
CYP108D1	*Novosphingobium aromaticivorans* DSM12444	3TKT		Aromatic hydrocarbon, e.g., phenanthrene hydroxylase	[751]
CYP164A2	*Mycobacterium smegmatis*	3R9B, 3R9C	Econazole	Binds fatty acids (C12–C18), Ortholog of M. leprae CYP (60%)	[412]
CYP153A7 (P450pyr)	*Sphingopyxis macrogoltabida*	3RWL		Hydroxylation of N-substituted pyrrolidines, piperidines, azetidines, 2-pyrrolidines and 2-piperidinones	[785]
CYP105N1	*Streptomyces coelicolor* A3(2)	3TYW, 4FXB		Monooxygenase involved in coelibactin synthesis	[169, 179]
CYP199A4	*Rhodopseudomonas palustris* HaA2	4DNZ, 4DO1, 4EGM, 4EGN, 4EGO, 4EGD	4-Methoxybenzoic acid, 4-ethylbenzoic acid, veratric acid, indole-6-carboxylic acid, 2-naphthoic acid	Oxidation of para-substituted benzoic acids and demethylation of 4-methoxybenzoic acid (83% identity to CYP199A2)	[778, 786]
CYP142A2	*Mycobacterium smegmatis*	3ZBY, 2YOO	Cholest-4-en-3-one	Cholesterol and cholest-4-en-3-one -3-one 26 oxidase	[367]
CYP125A3	*Mycobacterium smegmatis*	4APY		Cholesterol oxidase	[367]
CYP107B (HmtN)	*Streptomyces himastatinicus* ATCC 53653	4E2P		γ-Hydroxylation of an unusual piperazic acid (Pip) motif in himastatin biosynthesis	[172]
HmtT	*Streptomyces himastatinicus* ATCC 53653	4GGV		Regio- and stereospecific C2/C3 epoxidation of L-tryptophan indole ring and subsequent cyclization forming hexahydropyrroloindole in himastatin biosynthesis	[172]

Table 6.2 (continued)

P450	Organism	PDB codes	Ligands	Function	Refs
CYP163B3 (P450Sky)	*Streptomyces* sp. Acta 2897	4LOE, 4L0F, 4PXH	PCP-linked azole	3 successive β-hydroxylations of separate PCP-bound L-amino acid precursors in skyllamycin biosynthesis	[236, 252]
CYP152L1 (OleT)	*Jeotgalicoccus* sp. ATCC 8456	4L4O, 4L54	Arachidic acid	Fatty acid decarboxylase/hydroxylase (H_2O_2-dependent peroxygenase)	[575]
CYP1048A1 (TxtE)	*Streptomyces scabiei* 87.22	4L36	Imidazole	Direct nitration of L-tryptophan (using nitric oxide, dioxygen and NADPH) in thaxtomin biosynthesis	[278]
CYP1050A1 (RauA)	*Rhodococcus erythropolis* JCM 6824	3WEC	Aurachin RE intermediate (3-[2E,6E,9R)-9-hydroxy-3, 7, 11-trimethyldodeca-2,6,10-trien-1-yl]-2-methylquinolin-4(1H)-one)	Hydroxylation of aurachin RE intermediate (nitrogen atom in the quinolone ring) in aurachin RE biosynthesis	[346]
CYP51F1	*Saccharomyces cerevisiae* YJM789	4LXJ, 4KOF	Lanosterol, itraconazole	Lanosterol 14α-demethylase, full length structure with membrane-spanning helix	[467]
CYP154C5	*Nocardia farcinica* IFM10152	4JBT, 4J6C, 4J6C, 4J6B	Androstenedione, testosterone, progesterone, pregnenolone	16α-steroid hydroxylase	

ACP acyl carrier protein

and their diversity in microbes') [113–116]. In addition, several microbial P450s have evolved to form natural fusions with other accessory enzymes (including both redox and nonredox partners), with many of these novel P450 fusion proteins still to be isolated and characterized (see the section 'Microbial P450- (redox) partner fusion enzymes').

P450cam (CYP101A1) from *Pseudomonas putida* is probably the best-studied example of a bacterial P450, functioning in the hydroxylation of camphor as part of a pathway for degradation of the molecule as a carbon and energy source [117]. Similar roles are found in the related 5-*exo* camphor hydroxylases CYP101D1 and CYP101D2 from *Novosphingobium aromaticivorans* DSM 12444 [118, 119], as well as in the CYP108A1 α-terpineol hydroxylase (P450terp) from a *Pseudomonas* sp. [120] and in the cineole oxidizing CYP176A1 (P450cin) [38, 121, 122]. The crystal structures (Fig. 6.2), catalytic properties, and redox partner interactions of P450cam and related enzymes are discussed later in this chapter (e.g., see the section 'Diverse FD partners' in *Redox partner systems and their diversity in microbes*). In addition to terpenes, various microorganisms can utilize aliphatic alkanes as their sole carbon and energy source, with hydroxylation reactions often being important steps in their catabolism [123, 124]. Examples of alkane hydroxylating P450s are often members of the CYP153 family, including CYP153A6 from *Mycobacterium* sp. HXN-1500 which preferentially hydroxylates medium chain length (C6–C11) alkanes [125]; CYP153C1 from *N. aromaticivorans* [126]; and CYP153 enzymes from *Alcanivorax hongdengensis* A-11-3T [127] and *Acinetobacter* sp. EB104 [128]. A number of *Bacillus* P450s catalyze the oxidation of fatty acids. These P450s are discussed individually in more detail later in the chapter and include the well-characterized *Bacillus megaterium* P450 BM3 (CYP102A1), a model fatty acid-oxidizing flavocytochrome P450 (see the section 'Microbial P450-(redox) partner fusion enzymes') for which engineered variants have potential biotechnological applications [28, 129] (see the section 'Conclusions and future prospects'; Fig. 6.2). The peroxygenase P450 BS_{β} (CYP152A1) from *B. subtilis* catalyzes the β-hydroxylation of fatty acids using hydrogen peroxide in the 'peroxide shunt' pathway (see Fig. 6.4), as do other CYP152 peroxygenase family members from other microbes [130] (see the section 'P450 systems that bypass redox partner systems in the Redox partner systems and their diversity in microbes'). In addition, a further *B. subtilis* P450 (BioI or CYP107H1) catalyzes the oxidative cleavage of fatty acids linked to an acyl carrier protein (ACP) in the biosynthesis of pimelic acid, a biotin precursor [131] (see the section 'Nonredox partner proteins for microbial P450s in the Redox partner systems and their diversity in microbes'). Steroid hydroxylase activity is also recognized in *B. subtilis,* with CYP106A2 characterized as a steroid 15β-hydroxylase that oxidizes progesterone and 11-deoxycortisol, and for which activity can be supported by the eukaryotic adrenodoxin reductase (ADR) and adrenodoxin proteins [63]. In recent work, CYP106A2 was also shown to convert dehydroepiandrosterone (DHEA) into 7β-OH-DHEA, a human metabolite with proposed neuroprotective and anti-inflammatory properties [132]. The highly related CYP106A1 from *Bacillus megaterium* also catalyzes hydroxylation of the pentacyclic triterpene 11-keto-β-boswellic acid, with activity supported by flavodoxin and FD redox partners from the same bacterium [133].

Microbial P450s also have roles in the degradation of toxic compounds, a function more commonly associated with the xenobiotic metabolizing eukaryotic P450 enzymes, such as those in the human liver. CYP177A1 (XplA) is an unusual flavocytochrome P450 from *Rhodococcus rhodochrous* 11Y that is a natural fusion with a flavodoxin redox partner and which has an interesting role with biotechnological applications. CYP177A1 initiates the breakdown of the military explosive and recalcitrant environmental pollutant hexahydro-1,3,5-trinitro-1,3,5-triazene (RDX, Royal Demolition Explosive) and its nitroso derivatives by reductive denitration, with XplA orthologs restricted to a number of *Rhodococcus* spp. [134–137] (see the section 'P450 fusions to flavodoxin and FD proteins' under

Microbial P450-(redox) partner fusion enzymes). The CYP116s are another family of bacterial P450 enzymes that are involved in the degradation of toxic compounds. CYP116A1 from *Rhodococcus erythropolis* NI86/21 (along with its genetically adjacent redox partners) was shown to N-dealkylate the thiocarbamate herbicides EPTC (*S*-ethyl dipropylthiocarbamate) and vernolate (*S*-propyl dipropylthiocarbamate), and the triazine herbicide atrazine (1-chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine), conferring protection on crop plants against the application of these herbicides [138–140]. The orthologous CYP116B1 from *Cupriavidus metallidurans* [141] and CYP116B2 (P450-Rhf) from *Rhodococcus* sp. NCIMB 978 [142, 143] constitute a distinct class of P450 fusion enzymes with a P450 domain linked to a phthalate dioxygenase reductase (PDOR; see *Microbial P450-(redox) partner fusion enzymes*). CYP116B1 was shown to catalyze propyl chain hydroxylation of the herbicides EPTC and vernolate, with subsequent N-dealkylation in the case of vernolate [141]. CYP116B2 was shown to catalyze hydroxylation and O-dealkylation of several alkyl aryl ethers, with a preference for shorter-chain alkyl groups in these substrates [143]. Although the exact physiological functions of these enzymes remain to be determined, it appears likely that these fusion P450s may play a similar role to that seen for CYP116A1 in one or more detoxification reactions, including the oxidative degradation of herbicides such as EPTC. The CYP116B family may exemplify divergent evolution in microbial P450s, with the relevant ancestral *CYP* gene becoming fused with a novel *PDOR* redox module gene to provide better catalytic efficiency and (possibly) stability in the new fusion enzyme, thus enhancing competitiveness of the host microbe in a challenging environment.

It is increasingly clear that, in addition to the utilization of unusual carbon sources, many bacterial P450s are crucial for other physiological roles [144, 145]. Among the most obvious examples of these are *Streptomyces* and other actinomycete species, where various P450s have defined roles in the production of antibiotics and other natural products that have benefits to human and animal health, and uses in agriculture (Table 6.3). The metabolomes of *Streptomyces* spp. are a rich source of secondary metabolites that account for more than two thirds of the microbially derived antibiotics, and *Streptomyces* also produce enzymes that can transform xenobiotics of industrial and environmental importance (e.g., steroids in the soil) [146, 147].

6.2.3.1 Streptomyces P450s

The *Streptomyces* P450s are frequently integrated into biosynthetic operons for secondary metabolite pathways, with these operons containing the majority of the enzymes required for production and export of the antibiotic or other secondary metabolite product. Creation of new antibiotics, as well as improvements in the production of naturally occurring agents, can be effected by manipulating the genes in these pathways, and such methods were shown to be successful in the generation of new and improved *Streptomyces* compounds [148, 149]. Examples of the diversity of *Streptomyces*-derived natural products whose synthesis requires activity of P450 enzymes include pharmaceuticals such as: anticancer agents (e.g., quinomycin [150] and daunorubicin [151]); antitumor agents (e.g., epothilone [152], fostriecin [153] and staurosporine [154]); immunosuppressives (e.g., tautomycetin [155], FK506 [156] and rapamycin [157]); antiparasitic agents (e.g., avermectin [158]); insecticides (e.g., nikkomycin [159]); antifungals (e.g., pimaricin [160] and amphotericin [161]); and antibacterial agents (e.g., clavulanic acid [162], oleandomycin [163], and pikromycin [164]; Table 6.3). The *Streptomyces* often have *CYP* gene families unique within a particular species, but selected *CYP* family members are also common to various *Streptomyces* spp., particularly for the *CYP105* and *CYP107* family P450s which have multiple members detailed in the databases [19, 165]. The *CYP105* and *CYP107* families are generally associated with xenobiotic and secondary metabolism, and the characterization of growing numbers of these P450s illustrates their broad substrate specificities and diverse catalytic functions. These include vitamin D hydroxylation (CYP105A1) [166], steps in the synthesis

Table 6.3 Examples of selected actinomycete P450-derived drugs and natural products. The action of the drug and the P450 catalyzed steps during its biosynthesis are included, along with PDB codes for structurally characterized enzymes

Drug	Action	P450(s)	Organism	P450 functionalization(s)	PDB	Refs
Albaflavenone	Antibiotic	CYP170A1	*Streptomyces coelicolor*	2-step allylic oxidations of epi-isozizaene	3DBG, 3EL3	[203, 787]
Amphotericin B	Antifungal	CYP161A3 (AmphL) and CYP105H4 (AmphN)	*Streptomyces nodosus*	Likely polyketide oxidative tailoring reactions		[161, 788]
Aurachin RE	Antibacterial	CYP1050A1 (RauA)	*Rhodococcus erythropolis* JCM 6824	Hydroxylation of aurachin RE intermediate	3WEC	[338, 346]
Aureothin	Antifungal, antitumor, insecticide	CYP151A (AurH)	*Streptomyces thiolteus*	2x C7 deoxyaureothin oxidations and C9a oxidation with ring formation	3P3L, 3P3X, 3P3O, 3P3Z	[226]
Avermectin	Antiparasitic	CYP171A1 (AveE)	*Streptomyces avermitilis*	C6 and C8a avermectin algycone hydroxylation		[158]
Calicheamicin	Antitumor, antibiotic	CYP105W1 (CalE10) and CYP248A1 (CalO2)	*Micromonospora echinospora*	TDP-alpha-D-4-amino-4,6-deoxyglucose N-oxygenation and orsellinic acid hydroxylation	3BUJ (CalO2)	[230, 288, 291]
Carbomycin	Antibacterial	CYP107C1 (orfA)	*Streptomyces thermotolerans*	C12–C13 epoxidation of carbomycin B to make carbomycin C		[789, 790]
Chalcomycin		ChmH1	*Streptomyces bikiniensis*	C20 methyl macrolide hydroxylation		[308]
Clavulanic acid	Antibacterial	CYP105M1 (orf10)	*Streptomyces clavuligerus*	Possible clavaminic acid derivative epoxidase		[791–793]
Daunorubicin	Antitumor	CYP129A2 (doxA) and CYP131A2 (dnrQ)	*Streptomyces* sp. strain C5	C10, C13, C14 anthracycline glycone DNR precursor hydroxylations and likely aglycone core oxidation		[794–796]
Epothilone	Antitumor	CYP167A1 (P450 EpoK)	*Sorangium cellulosum*	Epothilone C and D epoxidation	1Q5D, 1Q5E, 1PKF	[152, 761]
Erythromycin	Antibacterial	CYP107A1 (P450 EryF)	*Saccharopolyspora erythraea*	6-Deoxyerythronolide B hydroxylation	E.g., 1OXA, 1Z8O	E.g., [34, 36, 773]
Filipin	Antifungal	CYP105P1and CYP105D6	*Streptomyces avermitilis*	C1- (CYP105D6), and C26- (CYP105P1) filipin hydroxylation	3ABB and 3E5J, 3E5K, 3ABA	[167, 168]
FD-891	Antitumor	GfsF	*Streptomyces graminofaciens*	C8-9 macrolide epoxidation then C10 hydroxylation		[310, 797]
FK506	Immunosuppressive	CYP122A4 (FkbD)	*Streptomyces tsukubaensis*	4-Electron C-9 FK506 precursor oxidation		[156]

Table 6.3 (continued)

Drug	Action	P450(s)	Organism	P450 functionalization(s)	PDB	Refs
Fostriecin	Antitumor	FosK	*Streptomyces pulveraceus*	C18 fostriecin hydroxylation		[153, 798]
Himastatin	Antibacterial	CYP107B (HmtN) and HmtT and HmtS	*Streptomyces himastatinicus*	Piperazic acid (Pip) motif γ-hydroxylation and C2/C3L-tryptophan epoxidation then cyclization to hexahydropyrroloindole, and biaryl aromatic coupling of depsipeptide monomers	4E2P (HmtN) and 4GGV (HmtN)	[172, 214, 215]
Mycinamycin	Antibacterial	CYP105L2 (MycCI) and CYP107E1 (MycG)	*Micromonospora griseorubida*	C14-Hydroxylation and C21 mycinamicin VIII methyl hydroxylase and C12/C13-macrolactone epoxidation	E.g., 2YGX, 2YCA, 2Y98, 2Y5Z (MycG)	[301, 305, 306, 311]
Nikkomycin	Insecticidal	CYP162A1 (NikQ)	*Streptomyces tendae*	Histidine β-hydroxylation to form nikkomycins X and I		[159, 799]
Novobiocin	Antibacterial	CYP163A1 (NovI)	*Streptomyces spheroides*	PCP-loaded tyrosine β-hydroxylation		[243, 336]
Oleandomycin	Antibacterial	CYP105F2	*Streptomyces peucetius*	Oleandomycin tailoring hydroxylation		[163, 170]
Pikromycin	Antibacterial	CYP107L1 (PikC)	*Streptomyces venezuelae*	12- and 14-carbon macrolactone e.g., narbomycin and YC-17 hydroxylation	E.g., 2BVJ, 2CD8, 2C7X	E.g., [208, 210, 708]
Pimaricin	Antifungal	CYP161A2 (PimD)	*Streptomyces natalensis*	4,5-Desepoxypimaricin epoxidation	2X9P, 2XBK	[216, 800]
Rapamycin	Immunosuppressive, antifungal and antitumor	CYP107G1 (rapN), CYP122A2 (rapJ), CYP122A3	*Streptomyces hygroscopicus*	Likely C9, C26, C27 and C32 rapamycin macrolactone hydroxylation		[157, 801]
Staurosporine	Antitumor	CYP245A1 (StaP) and CYP244A1 (StaN)	*Streptomyces* sp. tp-a0274	Aryl-aryl coupling of chromopyrrolic acid and C–N linkage of staurosporine aglycone and deoxysugar	2Z3T, 2Z3U, 3A1L (StaP)	[154] [802]
Skyllamycin	Antibacterial, immunosuppressive, cytostatic, and antiparasitic	CYP163B3 (P450Sky)	*Streptomyces* sp. Acta 2897	3 successive β-hydroxylations of separate PCP-bound L-amino acid precursors	4LOE, 4L0F, 4PXH	[236, 252]
Tautomycetin	Immunosuppressive	TauI/TmcR	*Streptomyces griseochromogenes*	C5 tautomycetin oxygenation		[155, 803]
Teicoplanin	Antibacterial	CYP165D3 (OxyE; and OxyA, B,C, D)	*Actinoplanes teichomyceticus*	Oxidative phenol coupling of FG-rings	3O1A, 3OO3	[239, 240, 314]

Table 6.3 (continued)

Drug	Action	P450(s)	Organism	P450 functionalization(s)	PDB	Refs
Tylosin	Antibacterial	CYP105L1 (TylHI, orf7), CYP113B1 (TylI), CYP154B1	*Streptomyces fradiae*	Likely C23 methyl lactone ring oxidase (CYP105L1) and C20 methyl O-mycaminosyl-tylactone hydroxylation (CYP113B1)		[804, 805]
Tirandamycin	Antibiotic	TamI	*Streptomyces* sp. 307–9	C10 oxidation of tirandamycin C to E, then C11-12 epoxidation and C18 hydroxylation		[309, 806]
Vancomycin	Antibacterial	(CYP165A3 OxyA), CYP165B3 (OxyB), (CYP165C4 OxyC), (CYP146A1, OxyD)	*Amycolatopsis orientalis* and *mediterranei*	β-tyrosine hydroxylase (OxyD) and oxidative phenol coupling of CD-(OxyB) and DE-rings (OxyA), and biaryl cyclization of AB-rings (OxyC)	1LFK, 1LG9, 1LGF (OxyB), 1UED (OxyC), 3MGX (OxyD)	[237, 248, 249, 318]

PDB Protein Data Bank, *PCP* peptidyl carrier protein

of the antibiotic filipin (CYPs 105D1 and P6) [167, 168], coelibactin siderophore biosynthesis (CYP105N1) [169], oleandomycin modification (CYP105F2) [170], biotin biosynthesis (CYP107H1) [64, 131], erythromycin biosynthesis (CYP107A1) [35], pikromycin biosynthesis (CYP107L1) [171], and himastatin biosynthesis (HmtT and HmtN (CYP107B)) [172] (Table 6.3). A number of CYPs are common in several *Streptomyces* spp., including those encoded by *CYP102, CYP154, CYP157,* and *CYP170* genes. Studies of some of these enzymes have revealed various interesting structural and catalytic features, as discussed below. However, thorough analysis of individual P450s in each *Streptomyces* species will be required to reveal all their true functions. The *Streptomyces* P450s characterized to date have diverse biochemical roles, and have evolved specialized functions to enable their host bacteria to exploit different environmental niches [67, 173].

At the time of preparation of this chapter, there were P450 crystal structures from 17 different *Streptomyces* species in the PDB (Table 6.2). These include a number of CYP105 family members with varied hydroxylase activities. Examples include CYP105A1 (P450 SU-1) from *Streptomyces grisoleus* (PDB 3CV9), which is a vitamin D_3 hydroxylase involved in the conversion of vitamin D_3 to its active form 1α,25-hydroxy vitamin D_3 (Fig. 6.5a) [174–176]. CYP105D6 and CYP105P1 (PDB 3ABB and 3ABA) from *S. avermitilis* perform filipin hydroxylations in the C1 and C26 positions, respectively, during biosynthesis of this polyene macrolide antibiotic [167, 168, 177] (Fig. 6.5B). CYP105N1 from *S. coelicolor* is a monooxygenase predicted to be involved in the biosynthesis of coelibactin, a siderophore with implications in zinc-dependent antibiotic regulation by *S. coelicolor* [169, 178, 179] (Fig. 6.5c). Interesting properties are also observed for *S. coelicolor* CYP154A1 [111, 180]. CYP154A1 performs an unusual cyclization reaction, coupling the C5 carbonyl and the C11–C12 double bond of a dipentaenone substrate (Fig. 6.5d) to form a product with an oxetane ring (Fig. 6.5e), doing so in the absence of NAD(P)H and redox partners and without the need for any net oxidation/reduction [111]. There are only a few examples of P450s that have the ability to catalyze molecular rearrangement reactions without external reducing equivalents. These include the CYP5A and CYP8A families involved in the production of thromboxane A2 and prostacyclin, respectively, through isomerization of prostaglandin endoperoxide (prostaglandin H2) in higher animals [181, 182]. In addition, the fatty acid hydroperoxide metabolizing CYP74 AOS are involved in the dehydration of linolenic acid 13-hydroperoxide (18:3ω3) during the early steps of jasmonic acid production in plants [181, 183–185]. In contrast to CYP154A1, where the exact reaction mechanism is unknown, these P450s are able to utilize their peroxide substrates directly in the generation of reactive species for both the enzyme and substrates during catalysis [181, 186]. The physiological role of CYP154A1 is not clear, although it may function in spore stabilization during the *S. coelicolor* growth cycle. In addition, the substrate pentaenone ring bears a resemblance to the *S. coelicolor* antibiotic methylenomycin C scaffold, although the presence of a biosynthetic pathway involving the pentaenone is yet to be established [111]. The 4-phenylimidazole-bound crystal structure of CYP154A1 reveals a closed conformation with some disorder in the central region of the I-helix, and with various amino acid residues observed in distinct positions [180]. The CYP154A1 heme is found in two orientations (related by a 180° flip) as also described for the *Mycobacterium tuberculosis* CYP121 [187], although whether there is any physiological/mechanistic relevance here, or influence on catalysis, remains unclear [180]. Interestingly, *CYP154A1* is located directly upstream of the uncharacterized *CYP157C1* gene (which encodes a P450 with no EXXR motif in a 'conservon' five-gene cluster repeated throughout the *S. coelicolor* genome) [42, 147]. Despite the retention of these conservons, there is no real clue as to their physiological relevance, and the substrate and product of CYP154A1 were determined through elegant metabolomic approaches rather than by identification of the function of a biosynthetic operon [111]. The second structurally characterized *S. coelicolor* CYP154 fam-

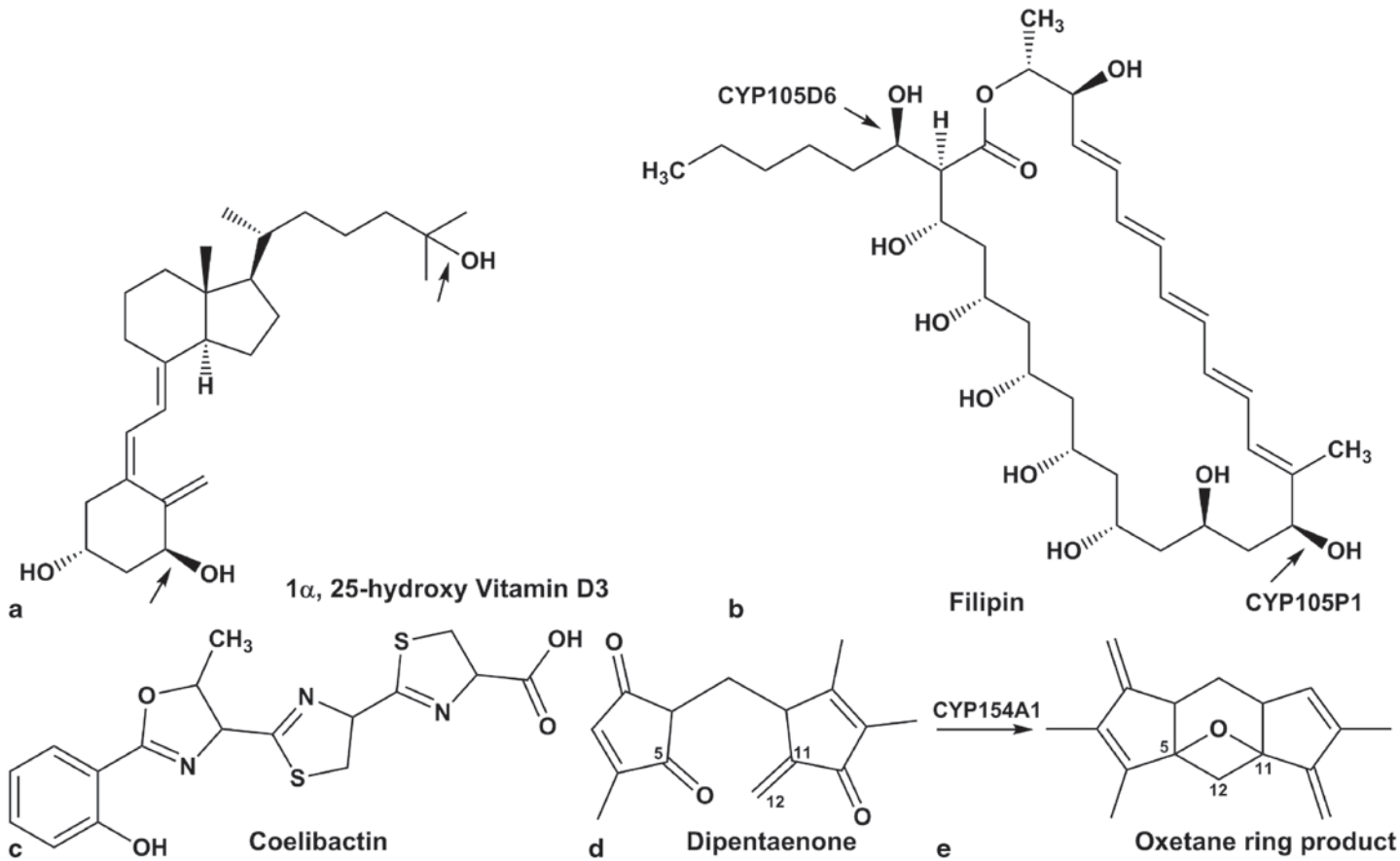

Fig. 6.5 Examples of selected P450-derived products from *Streptomyces* spp. **a** The *S. grisoleus* CYP105A1 conversion (*arrows*) of vitamin D_3 to its active form 1α, 25-hydroxy vitamin D_3 [848]. **b** The *S. avermitilis* CYP105D6- and CYP105P1-catalyzed C1 and C26 hydroxylations (*arrows*), respectively, during filipin biosynthesis [167]. **c** Coelibactin, a *S. coelicolor* siderophore with implications in zinc-dependent regulation of antibiotic synthesis. CYP105N1 performs an oxidative tailoring reaction during coelibactin biosynthesis, likely involving hydroxylation of the phenyl ring [169]. Finally, the unusual cyclization reaction catalyzed by *S. coelicolor* CYP154A1 involves coupling of the C5 carbonyl and the C11-12 double bond of a dipentaenone substrate (**d**) to form a product with an oxetane ring (**e**) in the absence of NAD(P)H [111]. NAD(P)H nicotinamide adenine dinucleotide phosphate

ily enzyme is CYP154C1 with 42% identity to CYP154A1. In contrast to CYP154A1, the CYP154C1 structure is in an open conformation in the ligand-free state (PDB 1GWI) [188]. A swinging movement of the FG helices and a reorganization of the BC loop are observed in comparison to CYP154A1, which results in the open conformation with a direct access pathway to the heme observed from the protein surface. CYP154C1 is also located adjacent to another uncharacterized P450 (CYP157A1, again lacking the EXXR motif) in an operon that does not contain nearby polyketide synthases or nonribosomal peptide synthetase (NRPS) gene sequences and which gives no obvious clue to the P450 function [42]. However, CYP154C1 was shown to possess the same activity as CYP107L1 (PikC) from *S. venezuelae,* which performs successive hydroxylations on the 12- and 14-membered ring macrocyclic lactones YC-17 and narbomycin [164] (discussed further below), although the true role of CYP154C1 in *S. coelicolor* is still to be determined [180, 188]. Additional members of the CYP154 family have been identified only in actinomycetes and were found to have a broad spectrum of substrate selectivity towards molecules of diverse size and chemical characteristics, and to have distinctive catalytic roles. CYP154A8 from *Nocardia farcinica* IFM10152 catalyzes hydroxylation of 7-ethoxycoumarin and the O-demethylation and subsequent *ortho*-hydroxylation of the medium-sized isoflavonoid formononetin via a daidzein intermediate [189]. Furthermore, compound screening studies have revealed a wide range of hydroxylation activities, mainly for long rod-shaped compounds as well as the stereo- and regio-selective hydroxylation of n-alkanes [190, 191]. CYP154C5, also from *N. farcinica* IFM 10152, was shown to possess 16-α hydroxylase activity with the steroids androstenedione, dehydroepiandrosterone, nandrolone, pregnenolone, progesterone, and testosterone [192, 193]. The crystal structures of CYP154C5 in complex with androstenedione, pregnenolone, progesterone and testosterone have been deposited recently in the PDB (PDB codes 4JBT, 4J6B, 4JSC, and 4JSD, respectively) with no citation available at the time of completion of this chapter. CYP154E1 from *Thermobifida fusca* YX displayed a broad substrate range following screening with diverse molecules ranging from heptanoic acid, 2,4,6-trimethyloctanoic acid, benzyl methyl sulfide, and nootkatone through to the largest substrate compound pergolide mesylate [190]. CYP154H1, also from *T. fusca* YX, catalyzes side-chain hydroxylation of small aromatic and arylaliphatic molecules such as ethylstyrene, ethylbenzene, styrene, and indole, as well as the S-oxidation of aromatic thioethers to their corresponding sulfoxides and sulfones [194].

The CYP158 family is so far restricted to the actinomycetes, with CYP158A enzymes found only in *Streptomyces* species. *CYP158B* genes have also been identified in the actinomycetes *Saccharopolyspora erythraea* NRRL23338 and *Saccharopolyspora spinosa*. However, the majority of genes in the *CYP158* family remain uncharacterized [19, 165]. The related *S. coelicolor* enzymes CYP158A1 and CYP158A2 catalyze aryl ring coupling reactions resulting in flaviolin dimerization/multimerization, producing differing products [195]. The polymerization of flaviolin(s) produces red-brown pigment molecules thought to provide the bacteria with protection from UV light [196, 197]. CYP158A2 is one of the few *S. coelicolor* P450s found within a clearly defined three-gene operon that produces the P450 substrate molecule flaviolin (Fig. 6.6a) in a process also involving a type III polyketide synthase and a naphthalene monooxygenase. CYP158A2 was shown to produce three isomers of biflaviolin and one triflaviolin from the mono-substituted flaviolin substrate [173, 195]. In contrast, CYP158A1 (which has 61% amino acid identity to CYP158A2) is not found in an apparent operon, but can produce 3,3′-biflaviolin and 3,8′-biflaviolin as its only flaviolin products and ultimately may have a different physiological role to CYP158A2 [195]. The structure of CYP158A2 revealed two flaviolin molecules bound in the active site (Fig. 6.6b), stacking with the heme cofactor and creating a kink in the I-helix and a repositioning of the FG helices to close the active site (PDB 1T93; Fig. 6.6c). The CYP158A2 heme cofactor is bound with the A-propionate

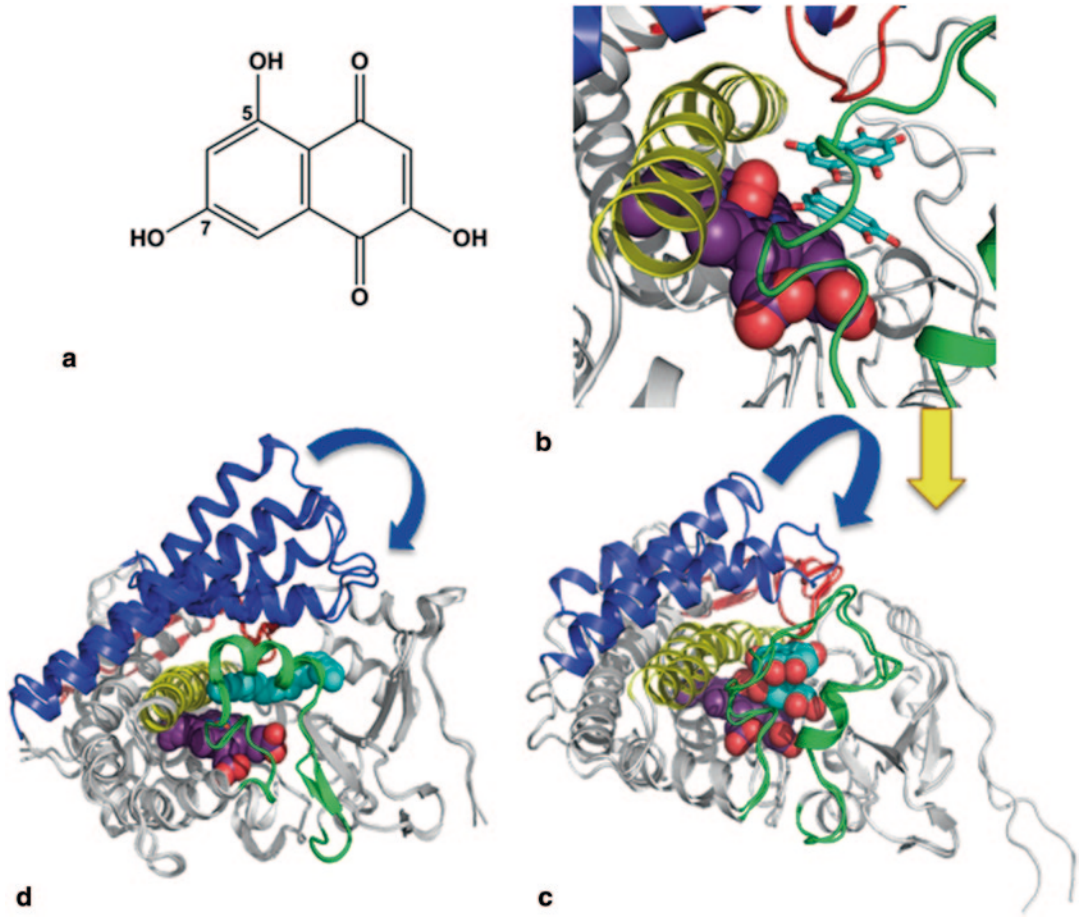

Fig. 6.6 CYP158A2 and its substrate flaviolin. The substrate flaviolin (**a**) is polymerized to form a *red-brown* pigment via P450-mediated aryl ring coupling. **b** The substrate-bound P450 structure contains two flaviolin molecules in the active site cavity and dioxygen bound to the heme iron (PDB 1T93). Structural rearrangements associated with substrate binding occur in P450 enzymes [198]. Panels **c** and **d**: An overlay of substrate-bound and substrate-free structures, respectively, for two distinct P450s: *S. coelicolor* CYP158A2 (**c**) (PDB 1T93 and 1S1F) [198] and the P450 BM3 heme domain (**d**) (PDB 1FAG and 2HPD) [271, 701]. In both cases, the FG helices reorient to wrap around the substrate, closing the active site (indicated by *blue arrows*). In addition, a portion of the CYP158A2 I-helix is seen in a distinct position upon substrate binding forming a kink resulting in the positioning of an ordered water molecule (WAT50; not shown) that hydrogen bonds with the 5-OH moiety of the proximal flaviolin. It has been postulated that WAT50 may also provide a hydrogen bond to the distal oxygen atom during catalysis [195]

moiety on the proximal side of the heme and it is suggested that this orientation allows a greater amount of space on the distal side of the heme, subsequently creating a larger active site cavity to allow binding of the bulky flaviolin substrate(s) [112]. CYP158A2 does not possess the conserved threonine (as described for P450cam, see above), with an alanine (Ala245) in this position, and its reaction is postulated to involve substrate assisted phenolic coupling with the flaviolin substrate (as described above for EryF above). The flaviolin C5 and C7 hydroxyl groups (Fig. 6.6a) are positioned to act as H-bond donors, stabilizing the proton pathway to the heme iron during catalysis [35, 195, 198, 199]. The CYP158 family P450s contain a highly conserved active site residue (Ile87 in CYP158A2) located on the BC loop. In CYP158A2, Ile87 points inwards within

the active site cavity interior and interacts with the second flaviolin molecule. Mutagenesis of this residue and crystallographic studies (PDB 3TZO 3TNK) show large differences in the overall BC loop topology, and reveal differing catalytic activities and dimerization products of the mutant enzymes, highlighting the importance of this active site residue in controlling the regioselectivity in formation of the flaviolin products [199]. The structure of CYP158A1 with two flaviolins bound revealed a distinctive mode of binding for the second substrate, which is found in the entrance to the substrate access channel and positioned too far away to allow dimerization to occur, despite evidence that this occurs in vitro (PDB 3NZ5) [195, 197, 198]. Structural comparisons with CYP158A2 show that CYP158A1 has a shorter I-helix with two fewer helical turns and no I-helix kink. These changes are accompanied by a longer loop between the H- and I-helices in CYP158A1. CYP158A1 possesses a unique lysine residue (Lys90) in place of the conserved CYP158 family isoleucine (Ile87 in CYP158A2). The bulkier side chain of Lys90 in CYP158A1 results in its orientation towards the surface, pointing away from the BC loop. In contrast to CYP158A2, mutagenesis of the CYP158A1 Lys90 has no apparent effect on catalytic activity or selectivity [199]. It is predicted that there will be movement of the second flaviolin substrate along with the FG helices and BC loop to accommodate substrate dimerization in CYP158A1 [195, 199]. Structural differences in active site topology and chemical composition likely account for the altered product profiles observed in CYP158A1 and CYP158A2, and for their potentially quite different biological roles. Recent studies used CYP158A2 to produce large amounts of a heme stable iron(IV) hydroxide species (compound II) and to highlight the importance of the cysteine thiolate axial ligand in the P450s' ability to oxidize C–H bonds. The CYP158A2 compound II was determined to have a basic p*K*a (11.9), and it was suggested that thiolate coordination is important in increasing the compound II p*K*a, consequently lowering the redox potential for the one electron reduction of compound I. Theoretical studies indicated that the elevated p*K*a substantially decreases the rate of oxidation of surrounding amino acids to favor specific oxidation of the substrate. During C–H activation catalysis, it also appears likely that a solvent exposed CYP158A2 tyrosine residue (Tyr352) adjacent to the cysteine thiolate ligand (Cys353) provides a reducing equivalent that initiates the conversion from compound I (Cys–S–Fe(IV)=O) to compound II (Cys–S–Fe(IV)–OH) that favors C–H bond oxidation [200].

An intriguing *S. coelicolor* enzyme is the bifunctional CYP170A1, which was initially classified as a CYP51, but renamed once shown to be devoid of demethylase activity and to have low similarity to other CYP51 enzymes [67]. CYP170A1 is in a two-gene operon adjacent to a sesquiterpene cyclase that is involved in the ionization and successive isomerization of farnesyl diphosphate to the novel compound *epi*-isozizaene [201]. CYP170A1 converts *epi*-isozizaene via albaflavenol intermediate(s) to the sesquiterpene single ketone antibiotic albaflavenone by two successive allylic oxidations (Fig. 6.7a) [202]. Surprisingly, CYP170A1 was also discovered by gas chromatography/mass spectrometry (GC/MS) analysis to produce farnesene isomers (Fig. 6.7b) and was subsequently found to possess an additional terpenoid synthase activity in presence of Mg^{2+} alone (i.e., in a reaction not involving redox partners or reducing equivalents), generating a mixture of farnesene isomers from farnesyl diphosphate [203]. The terpenoid synthase activity is optimal at acidic pH (5.5–6.5), whereas the P450 oxygenase activity in the production of albaflavenone is most efficient at a neutral to basic pH (7.0–8.2), perhaps conferring an environmental control over the distinct activities. The crystal structure of CYP170A1 was determined in the ligand-free and *epi*-isozizaene-bound forms (PBD 3DBG and 3EL3, respectively) and confirmed the unusual bipartite function with a terpene synthase-like active site contained within the conventional P450 structure. This terpene synthase site is situated in the α-helical domain and forms a discrete, but slightly disordered, four-helical bundle located within the C-, H-, I-, and L-helices, and is internally lined with various hydrophobic amino acids in its cavity

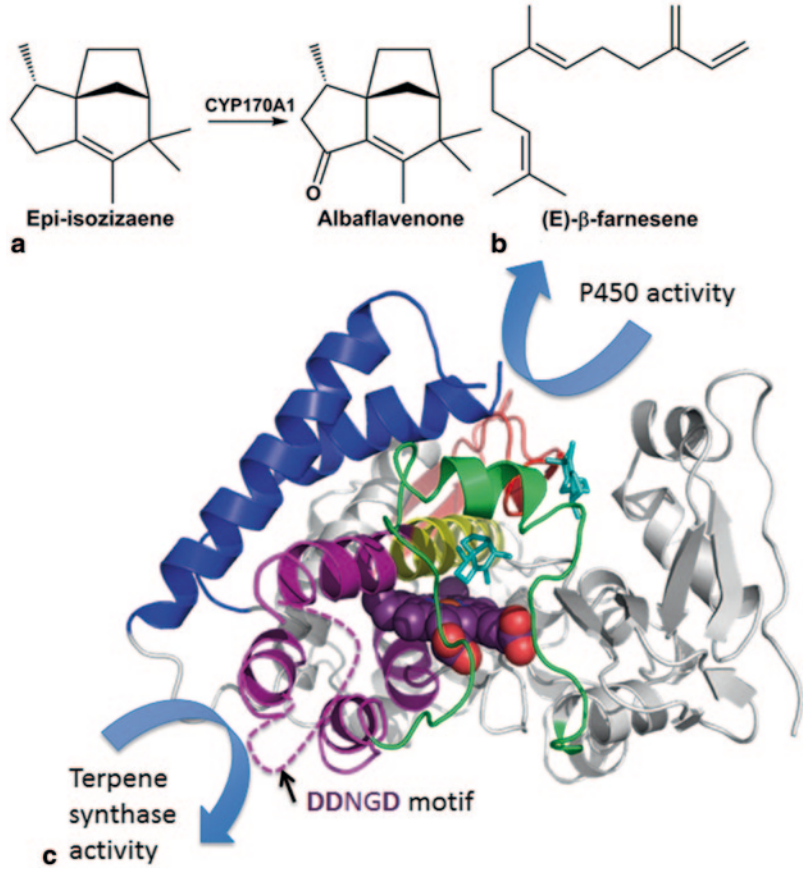

Fig. 6.7 A case of moonlighting. The *S. coelicolor* CYP170A1 converts *epi*-isozizaene via albaflavenol intermediate(s) to the sesquiterpene single ketone antibiotic albaflavenone by two successive allylic oxidations (**a**) [202]. A secondary terpenoid synthase role was also discovered to produce farnesene (**b**) [849]. The structure of CYP170A1 is shown in (c) (PDB 3DBG), with the different activities indicated by *blue arrows*. The non-P450 terpene synthase activity is believed to reside at the N-terminal region of the I-helix (depicted in *purple*), centered around the Mg^{2+}-binding loop containing the DDNGD motif (disordered in the crystal structure) [203]

(Fig. 6.7c) [203]. Terpene synthases usually possess a six-helical barrel [204]. However, an overlay of the structures of CYP170A1 and terpene synthase enzymes displays a high level of similarly in the structural shape and the amino acids lining the cavities of the synthase units [203]. Comparisons of the CYP170A1 ligand-free and *epi*-isozizaene-bound structures with that of P450cam (CYP101A1) confirm structural features consistent with typical P450 topology [24, 112]. It is thought that CYP170A1 may undergo structural rearrangements to promote terpene synthase activity over P450 monooxygenase function, and to further order the helical farnesyl diphosphate active site. This conformational change may allow CYP170A1 to utilize one distinct activity over another, consistent with selectivity over different pH ranges [203]. CYP170A1 is one of the more diverse classes of *Streptomyces* P450, with rather low amino acid similarity to other characterized P450s. The CYP170 class has been investigated in other *Streptomyces* species [205, 206]. *S. avermitilis* possesses a similar CYP170A2 which is located adjacent to a sesquiterpene cyclase and was shown in vitro to produce albaflavenone from *epi*-isozizaene, as described for CYP170A1. Moreover, a novel oxidized *epi*-isozizaene metabolite was identified

and believed to be derived from the CYP170A2 intermediate (4*S*)-albaflavenol to produce the oxidized metabolite 4β,5β-epoxy-2-epi-zizaene-6β-ol. This reaction is most likely catalyzed by CYP170A2-dependent epoxidation, although the action of an additional unidentified P450 or epoxidase in the *S. avermitilis* genome cannot be ruled out [205]. Genome mining has also revealed the presence of the sesquiterpene cyclase/*CYP170*-like gene pairings in nine further *Streptomyces* bacteria (*S. albus* J1074, *S. lividans* TK24, *S. ghanaensis* ATCC14672, *S. griseoflavus* Tu4000, *S. sviceus* ATCC29083, *S. viridochromogenes* DSM40736, and *Streptomyces* spp. E14, SPB74 and SPB78), *with* a high degree of genomic conservation and synteny in the surrounding genes. The production of *epi*-isozizaene was detected in vivo in *S. ghanaensis, S. lividans* and *S. albus* following GC/MS studies of mycelia of each organism. Albaflavenone was detected in *S. viridochromogenes, S. griseoflavus, S. ghanaensis,* and *S. albus,* and the intermediate albaflavenols were detected in the latter organism [205, 206]. Furthermore, CYP170B1 from S. *albus* was found to produce albaflavenone from *epi*-isozizaene in vitro, but was unable to produce farnesene from farnesyl diphosphate, unlike the terpenoid synthase role identified in the *S. coelicolor* CYP170A1 ortholog. Structural analysis revealed the absence of key Mg^{2+}-binding amino acids, essential for the farnesene synthase activity, and thus explained the loss of the bifunctional enzymatic properties of CYP170B1. The CYP170 enzymes in *Streptomyces* sp. SPB74 and SPB78 also lack the farnesene synthase amino acids and are postulated to be members of the monofunctional *epi*-isozizaene hydroxylating CYP170B family [206].

CYP107L1 (PikC) from *S. venezuelae* is involved in the macrolide antibiotic pikromycin biosynthetic pathway and is probably the best-studied biosynthetic P450 [149, 164, 207]. PikC has specificity for both 12- and 14-carbon-membered rings of macrolactones, and has regio- and stereospecific oxygenase activities to enable production of different macrolide products [208]. PikC catalyzes the C12 hydroxylation of the 14-membered ring narbomycin to produce the ketolide pikromycin [154, 164] (Fig. 6.8a), and can perform a C14 hydroxylation to produce neopikromycin, as well as the dihydroxylation to produce novapikromycin, albeit to a very small extent [171]. PikC also catalyzes the monohydroxylation of the 12-membered ring YC-17 at either the C10 or C12 positions to produce methymycin and neomethymycin, respectively (Fig. 6.8b) [164]. The dihydroxylation of YC-17 produces novamethymycin via the methymycin precursor, which is also detected at very low levels [209]. The structures of PikC in the ligand-free and substrate-bound forms (PDB 2BVJ, 2C7X, 2CD) display conformational changes between open and closed conformations and highlight movements of the F and G helices as well as flexibility in the FG and BC loops that presumably allow entry and exit of the substrates and products. Interestingly, the ligand-free structure encompasses both open and closed conformations within two molecules in an asymmetric unit. The FG region adopts slightly differing positions depending on the ligand bound in the active site of PikC [208, 210]. An interesting feature observed in the PikC crystal structures is that the substrates are anchored in the upper part of the active site by the desosamine sugar moiety of narbomycin and YC-17 at different positions, forming a substrate-specific-binding pocket (Fig. 6.8c). A substrate-specific salt bridge is also formed between the C3′ dimethylamino group of the deoxysugar substituent and PikC glutamate residues (Glu85 for narbomycin binding and Glu94 for YC-17), with hydrogen-bonding networks formed with other amino acids on the BC loop, as confirmed by mutagenesis studies. These desosamine *N,N*-dimethylamino salt linkages and binding pockets, as well as unspecific hydrophobic interactions between active site amino acids and the macrolide portion of the substrate, play important roles in regio- and stereo-selectivity of substrate oxidation [208, 210, 211]. Substrate anchoring and engineering strategies have been employed to enable PikC-catalyzed hydroxylation(s) on nonnatural substrates fused with a desosamine sugar moiety using an engineered PikC (the D50N mutant enzyme), with the ultimate aim of harnessing PikC for the

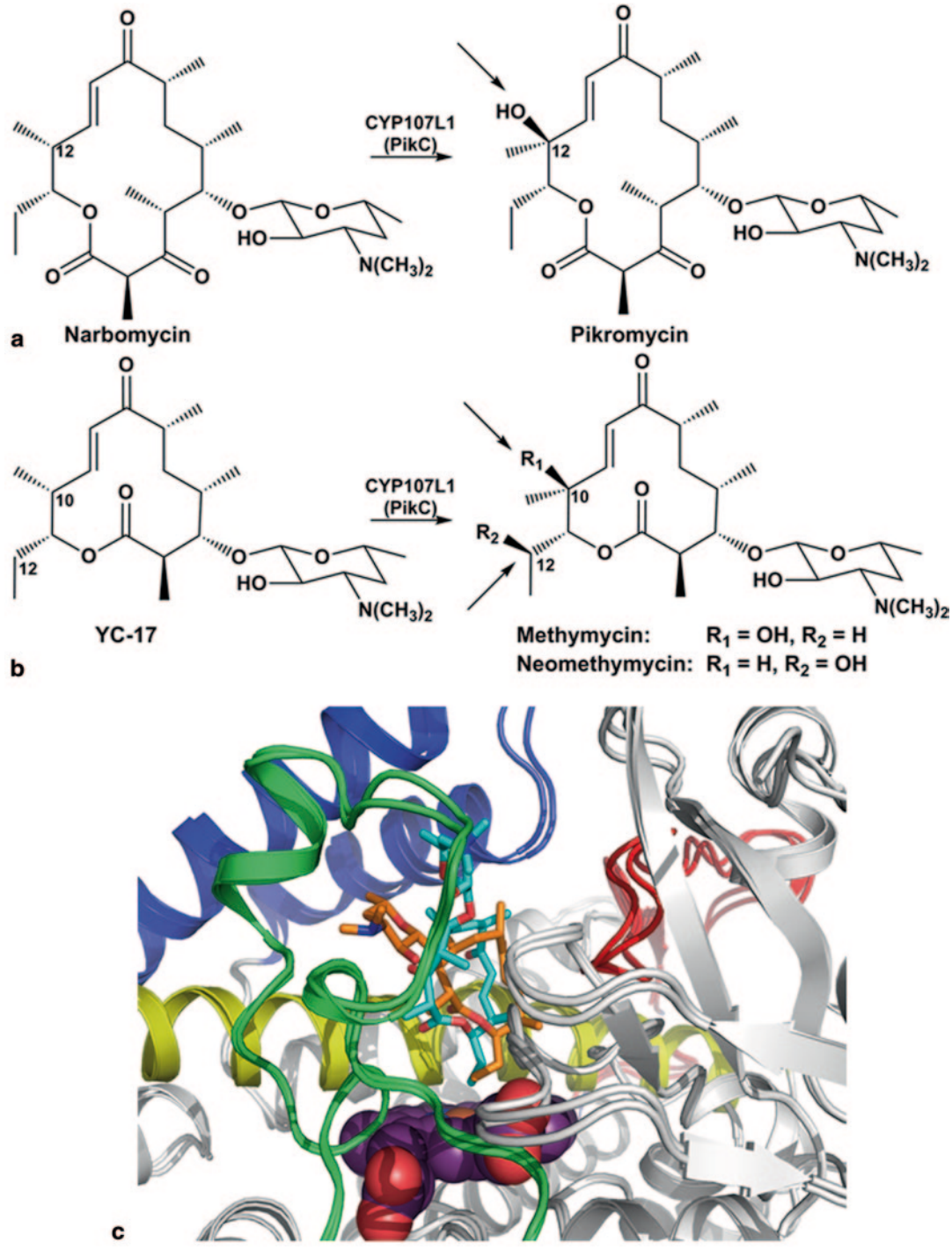

Fig. 6.8 Reactions catalyzed by PikC (CYP107L1). The scheme shows the PikC-catalyzed C12 hydroxylation of narbomycin to the ketolide pikromycin (**a**) and the C10 or C12 monohydroxylation of YC-17 to produce methymycin and neomethymycin, respectively (**b**). Positions of hydroxylation are shown by *arrows* [164]. **c** An overlay of P450 PikC bound to YC-17 (shown with *orange* carbons) and narbomycin (shown with *cyan* carbons; PDB 2CD8 and 2C7X) [208]

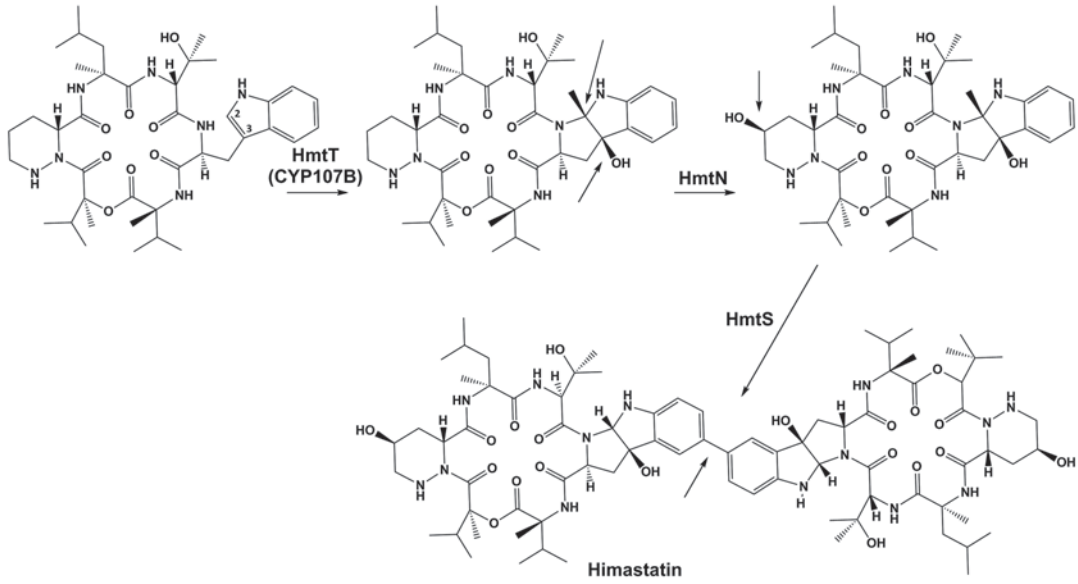

Fig. 6.9 P450-dependent reactions in the synthesis of the antibiotic himastatin. Himastatin biosynthetic reactions catalyzed by the P450s HmtT, HmtN, and HmtS are shown (with *arrows* highlighting the positions of the P450 reactions) [172, 214]. The scheme shows the HmtT-catalyzed regio- and stereospecific epoxidation of the C2/C3 double bond of the indole ring derived from *L*-tryptophan, and the subsequent cyclization to form the hexahydropyrroloindole moiety; the γ-hydroxylation of the piperazic acid (Pip) motif catalyzed by HmtN; and the biaryl aromatic coupling between cyclic depsipeptide monomers catalyzed by HmtS to create the active dimer form of himastatin

production of novel antibiotics, albeit likely with some limitations in ability to synthesize novel substrates and regulate enzyme regio-selectivity [211]. Improvements in PikC regio- and stereoselectivity were recently achieved with a similar substrate engineering approach, whereby the substrate desosamine was replaced with varied synthetic N, N-dimethylamino anchoring groups. It was demonstrated that the structure of the anchoring group can control regio-selectivity of the PikC C–H bond oxygenation [212]. Although pikromycin itself is not clinically used as an antibiotic, it has the potential to serve as a scaffold for the production of new macrolide therapeutic compounds [213]. These studies highlight the potential of PikC and other microbial biosynthetic P450s as biocatalysts for the development and production of new improved antibiotics and other secondary metabolites.

Three *S. himastatinicus* ATCC 53653 P450s (HmtT, HmtN, and HmtS) are involved in the synthesis of the antibiotic himastatin, a novel cyclohexadepsipeptide dimer that inhibits growth of Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) [214]. Himastatin has several tailoring groups, such as hydroxypiperazic acid and hexahydropyrroloindole moieties, attached to a depsipeptide ring with an unusual symmetry consisting of peptide residues in alternate *D*- or *L*-conformations [215]. The gene cluster for himastatin biosynthesis contains several NRPSs that are involved in peptide formation, as well as the P450s HmtT, HmtN, and HmtS. The three P450s share a 51–55 % level of identity and are likely members of the CYP107B family, but have distinct and novel roles in the oxidative tailoring of himastatin (Fig. 6.9) [214]. HmtT catalyzes the regio- and stereospecific epoxidation of the C2/C3 double bond of the indole ring derived from *L*-tryptophan, and subsequent cyclization reactions to form the tricyclic moiety hexahydropyrroloindole. HmtN catalyzes

the regio- and stereo-selective γ-hydroxylation of an unusual piperazic acid (Pip) motif. HmtS is involved in the biaryl aromatic coupling between cyclic depsipeptide monomers, catalyzing regio-selective C–C bond formation. This reaction is essential for himastatin activity as the depsipeptide monomer is inactive [172, 214]. The structures of HmtT and HmtN have been solved in ligand-free forms (PDB 4GGV and 4E2P, respectively). The HmtT structure reveals a remarkably long FG loop situated perpendicular to the heme plane and entering the active site cavity. The extra residues in this FG region are evident from amino acid alignments with other P450 enzymes and show notable differences within the himastatin P450s. The HmtT F and G helices are kinked as a result of the extra amino acid residues in this region. This unusual FG loop conformation is stabilized via hydrogen-bonding networks between Arg179/Asp66 and Phe165/Gln76. It was postulated that the long FG loop may form a significant part of the binding site in order to accommodate the large substrate size and to stabilize substrate orientation during the successive catalytic steps. Unlike HmtT, the structure of HmtN reveals relatively straight FG helices, similar to a number of other P450s in the PDB, and in a different orientation to the FG helices in HmtT [24]. In comparison to HmtT, the HmtN helices have moved laterally, resulting in an enlarged substrate access chamber to the active site. Despite HmtT and HmtN having a very similar substrate, this molecule would have to be oriented in opposing conformations to facilitate the P450-mediated reactions at different regions of the cyclohexadepsipeptide backbone. The variation in FG regions in the HmtT and HmtN structures presumably allows for these differing substrate configurations. HmtN does not possess the conserved threonine involved in oxygen activation, instead having a leucine (Leu244) in this position. The active site of HmtN has several ordered water molecules that form a hydrogen-bonding network, and it is possible that this network may assist proton delivery during catalysis [172]. CYP161A2 (PimD) from *S. natalensis* ATCC27448 is an epoxidase P450 involved in the biosynthesis of the antibiotic pimaricin (also called natamycin) [216]. Pimaricin is a 26-membered polyene macrolide that is commonly used to treat fungal keratitis (corneal infections) as well as being utilized as a food preservative to prevent mold contamination of cured meats and cheeses [217]. The large pimaricin biosynthetic gene cluster identified the PimD P450 as the final biosynthetic enzyme that catalyzes the epoxidation between the C4 and C5 double bond of 4,5-desepoxypimaricin to produce pimaricin (Fig. 6.10a) [160, 217]. The CYP161 family is restricted to the *Streptomyces,* with orthologous genes found only in species closely related to *S. natalensis* [19]. The structures of PimD in the ligand-free and substrate-bound forms (PDB 2X9P and 2XBK, respectively) reveal structural changes upon 4,5-desepoxypimaricin binding and give insights into the catalytic mechanism of PimD [216]. Comparisons of these PimD structures show the FG loop moves toward the active site on binding of 4,5-desepoxypimaricin, allowing interactions with the substrate. This is accompanied by an inward reorientation of the BC loop that is subsequently accommodated by an outward movement of the C-helix to allow the substrate-induced structural changes. The β-sheet 3 becomes more ordered in the substrate-bound form and closes over the active site, allowing an interaction with the pyranosidic ring of the mycosamine moiety of 4,5-desepoxypimaricin. PimD also lacks the conserved catalytic threonine, although does possess a serine residue (Ser238) in this position. However, the Ser238 side chain is rotated towards the interior of the I-helix, forming a hydrogen-bonding interaction with Ala234, and is thus unable to assist in proton delivery in this conformation [216]. PimD is thus a likely example of substrate-assisted epoxidation, whereby the 4,5-desepoxypimaricin C7–OH group is positioned to act as the proton donor for the epoxidation reaction, similar to that described above for EryF [34, 36, 216]. The PimD epoxidation reaction is thought to proceed via compound 0 (as opposed to compound I) with peroxo and hydroperoxo intermediates, the latter proposed to act as an oxidant for the insertion of the hydro-

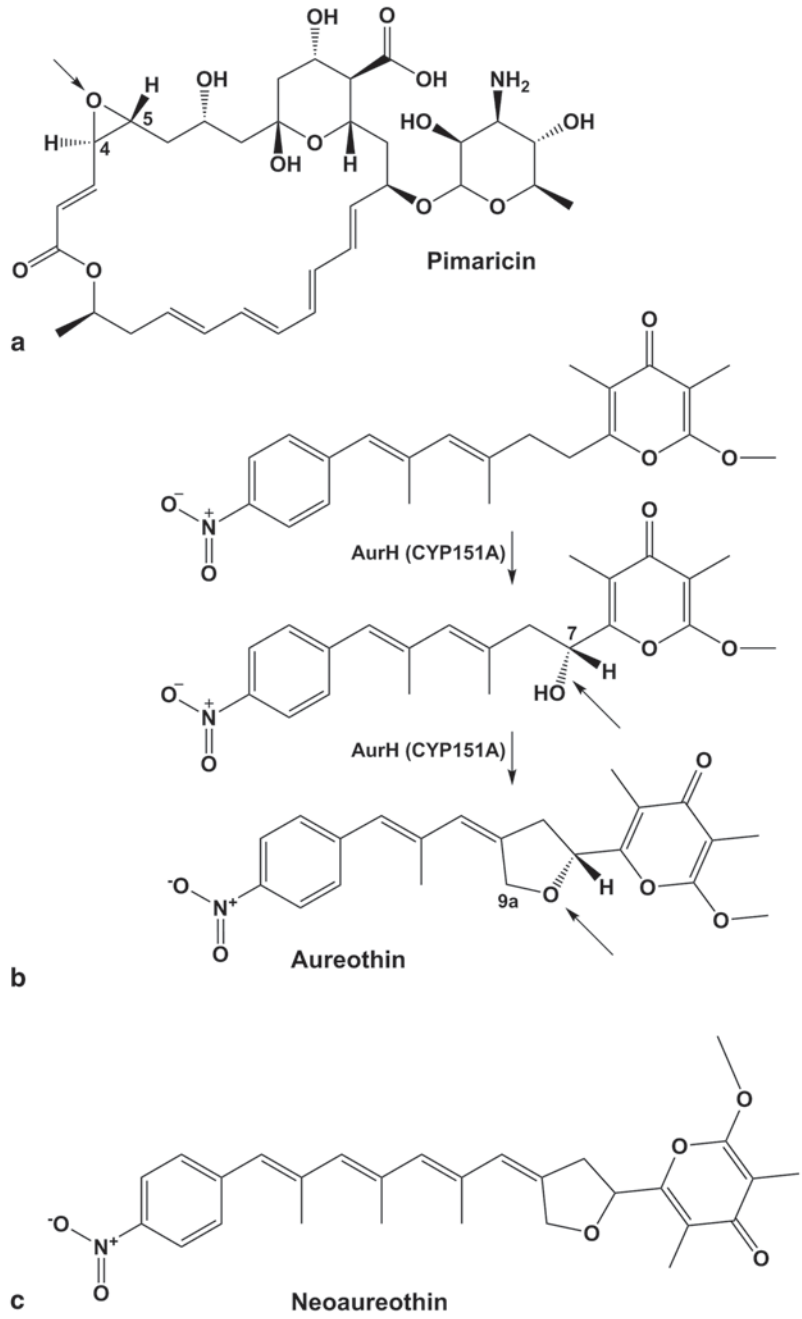

Fig. 6.10 P450 reactions in the synthesis of pimaricin, aureothin and neoaureothin. **a** The PimD-catalyzed epoxidation between the C4 and C5 double bond (*arrow*) of 4,5-desepoxypimaricin to produce pimaricin [216]. **b** Production of aureothin catalyzed by AurH (CYP151A), showing the successive P450-dependent oxidations (*arrows*) involving the C7 hydroxylation of deoxyaureothin to produce a (7*R*)-7-hydroxydeoxyaureothin intermediate, and oxidation at C9a that mediates O-heterocyclization to form aureothin [225, 226]. **c** The structure of the related antibiotic neoaureothin, where the corresponding reactions are catalyzed by the AurH orthologs NorH and SpnH in *S. orinoci* HKI-260 and *S. spectabilis,* respectively [227].

peroxo oxygen atom into the C4/C5 double bond on 4,5-desepoxypimaricin [149, 216].

The CYP151 family is actinomycete specific with a small number of orthologous genes identified in nonpathogenic *Mycobacterium* and *Streptomyces* species [19]. CYP151A (AurH) *from S. thioluteus* is involved in the production of the metabolite aureothin (Fig. 6.10b), a rare nitro-substituted polyketide with antifungal, insecticidal and antitumor activities [218–220]. The biosynthesis of aureothin involves a gene cluster with an unusual *p*-nitrobenzoate (PNBA) moiety that is derived from chorismate via a PNBA synthase and an *N*-oxidation reaction. An unusual nonlinear type I polyketide synthase extends PNBA with five successive (methyl)-malonyl-CoA moieties to form the polyketide backbone. Two successive tailoring reactions are then performed by an O-methyltransferase that catalyzes pyrene ring methylation (AurI) and a P450 (AurH) [221]. CYP151A (AurH) is an interesting multifunctional enzyme that is involved in the formation of a five-membered exomethylene tetrahydrofuran ring [222, 223]. This homochiral ring is responsible for the rigidity of the carbon backbone that is an essential component for the activity of aureothin [222, 224]. AurH performs two successive oxidations, initially catalyzing the asymmetric C7 hydroxylation of deoxyaureothin to produce a (7*R*)-7-hydroxydeoxyaureothin intermediate, immediately followed by a second oxygenation at C9a that mediates O-heterocyclization to form aureothin (Fig. 6.10b) [225, 226]. Orthologous P450s NorH and SpnH were identified in *S. orinoci* HKI-260 and *S. spectabilis,* respectively. These genes mediate a similar tetrahydrofuran ring formation of the anti-HIV and anti-malarial compound neoaureothin (spectinabilin; Fig. 6.10c), which differs only in the diene moieties, affecting the length of the polyketide backbone compared to the shorter aureothin. Interestingly, the otherwise near-identical neoaureothin biosynthetic pathways containing NorH and SpnH are transcriptionally regulated by activator proteins from different families (ArsR and AfsR, respectively). The *S. spectabilis* (SpnH-containing) neoaureothin biosynthetic gene cluster is more closely related to that for *S. thioluteus* aureothin synthesis than is the *S. orinoci* (NorH-containing) cluster, and phylogenetic analysis indicated that the aureothin pathway likely evolved from deletion of genes in the SpnH cluster [227, 228]. Basic Local Alignment Search Tool (BLAST) searches identify another P450 with high homology to AurH from *S. scabrisporus,* but it remains to be determined whether this organism can also make a nitroaryl-substituted polyketide involving the P450. The structure of AurH was determined in the ligand-free and inhibitor (ancymidol)-bound forms (PDB 3P3L and 3P3Z), as well as in forms with two N-terminal extensions derived from protein purification constructs that interact differently with the protein structures (PDB 3P3O and 3P3X) [226]. Ligand-free AurH displays a relatively open conformation, with some changes observed at the N-terminal and FG loops compared to its closest structural relatives in the CYP107 family, and with an interaction between the β2 loop and I-helix. An interesting difference is seen with some additional AurH residues located in the B region, forming an unusual, rigid two-helix bundle (helices B2 and B2′) that closes over the active site cavity [226]. This new helical bundle is situated in the place of the conventional loop/random coil region that contains a solvent-filled substrate access channel seen in the majority of P450 structures in the PDB (Fig. 6.11a) [24, 229]. A similar bundle has also been observed solely in the *Micromonospora echinospora* P450 CalO2 (see below; Fig. 6.11b) [230] and, by comparison, the AurH helical moiety is kinked toward the N-terminus and enlarges the closed active site cavity, presumably a reflection of the different substrate specificities for the CalO2 and AurH P450s. The structure of the ancymidol inhibitor-bound form reveals a transition from the open to a closed confirmation accompanied by changes in the organization of the FG loop and with the two-helix bundle moving closer into the active site cavity, and the β2 loop reorientating away from the I-helix and approaching towards the FG loop. Modeling studies revealed potential active site residues involved in substrate binding, and mutagenesis studies in AurH confirmed important roles for Phe89, Gln91, and Thr239 in substrate binding

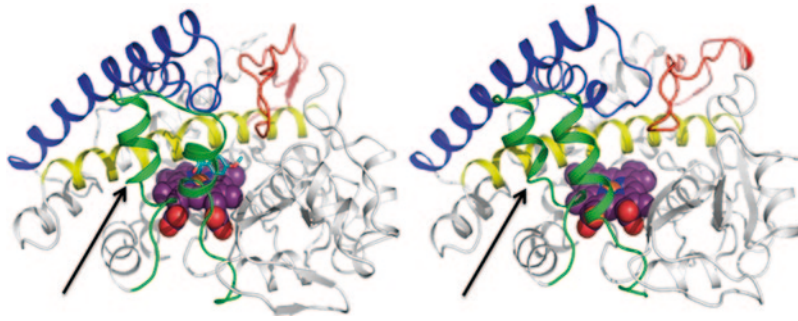

Fig. 6.11 Structures of the antibiotic pathway P450s AurH and CalO2. A structural comparison is shown between the ancymidol inhibitor-bound P450 AurH (*left*; PDB 3P3Z) [226] and the ligand-free P450 CalO2 involved in orsellinic acid oxidation (*right*; PDB 3BUJ) [230]. The additional B region two-helix bundle is shown in *green* (indicated by an *arrow*) and is postulated to be involved in carrier protein partner binding

and catalytic efficiency [226]. It will be interesting to see how the structure of AurH adapts to substrate binding and how this enables it to perform successive oxidations to produce the unusual tetrahydrofuran ring in future structural studies. Aureothin derivatives have been produced via manipulation of genes in the biosynthetic pathway, and AurH subsequently tailors these derivatives. These include a pyran analog aureopyran derived from AurH-dependent oxidation of the nonnatural substrate deoxyisoaureothin, and other aureothin derivatives produced by a mixture of synthetic and enzymatic steps [224, 231, 232]. The in vivo activities of these analogs have yet to be tested, but their synthesis highlights the capabilities of AurH as a biosynthetic enzyme and its potential to expand the rare aureothin class of nitro-polyketide drugs.

CYP163B3 (P450sky) from *Streptomyces* sp. Acta 2897 is involved in the biosynthesis of the cyclic depsipeptides skyllamycin A and B, with antibacterial, immunosuppressive, cytostatic, and antiparasitic properties [233]. Skyllamycin A has been isolated from different *Streptomyces* strains and is a potent inhibitor of the platelet-derived growth factor (PDGF) signaling pathway that is involved in important processes such as cellular proliferation and migration [234, 235]. The structure of skyllamycin has an unusual α-hydroxylated glycine residue, an N-terminal cinnamoyl side chain, and three β-hydroxylated amino acids ((*2S,3S*)-β-hydroxyphenylalanine, (2S,3S)-β-hydroxy-O-methoxytyrosine and (*2S,3S*)-β-hydroxyleucine) (Fig. 6.12). Interestingly, P450sky is responsible for the catalysis of all three β-hydroxylation reactions, found within domains 5, 7, and 11 on the skyllamycin peptide backbone, respectively [233, 236]. P450sky was shown to have unusual properties in catalyzing stereospecific β-hydroxylation reactions of three different *L*-amino acid substrates bound to peptidyl carrier protein (PCP) domains of the skyllamycin NRPS, with discrete selectivity for the prescribed PCP domain [236]. Direct interactions between P450s and NRPSs have also been described for the *Amycolatopsis* spp. CYP165 enzymes involved in the biosynthesis of the glycopeptide antibiotics vancomycin, balhimycin (using CYP164A3 (OxyA), CYP165B3 (OxyB), CYP165C4 (OxyC), and CYP146A1 (OxyD), and teicoplanin (additionally using CYP165D3 (OxyE)). However, in contrast to the triple hydroxylase role of P450sky, these P450s were shown to catalyze reactions with single PCP-bound substrates, and are discussed in more detail below [237–240]. The CYP165 family members are found only in certain *Streptomyces* spp., *Amycolatopsis* spp., and *Actinoplanes* spp. [19, 165]. The P450sky-containing CYP163 family is more diverse in the actinomycetes with many members in different *Streptomyces* spp. and in the unusual aminocoumarin-producing actinobacterium *Catenulispora acidiphila* [19, 165, 241, 242]. A *CYP163* gene orthologous

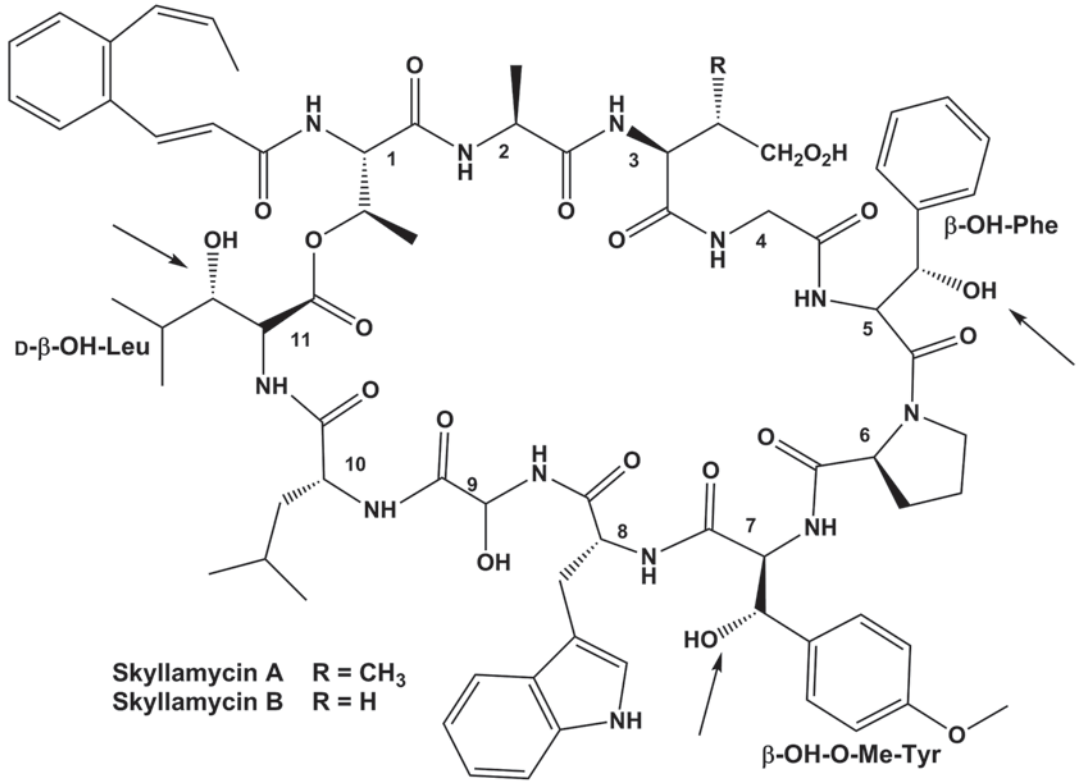

Fig. 6.12 The structure of the cyclic depsipeptide skyllamycin. Skyllamycin is a potent platelet-derived growth factor (PDGF) signaling pathway inhibitor. The core amino acid domains of skyllamycin are numbered. The three P450sky-mediated hydroxylations in domains 5, 7, and 11 are highlighted by *arrows* [233]

to P450sky was also identified in the biosynthetic pathway of the aminocoumarin novobiocin in *S. spheroides,* containing the tyrosine β-hydroxylase NovI (CYP163A1) that generates (2*S*,3*R*)-β-OH-tyrosine from PCP-loaded tyrosine (Fig. 6.13a) [243]. Further, CYP163 members have been identified in the biosynthetic pathways of the aminocoumarins chlorobiocin (CloI, CYP163A2) (Fig. 6.13b) [244], coumermycin (CumC, CYP163A) [245] (Fig. 6.13c) and simocyclinone (SimI, CYP163A3; Fig. 6.13d) [246, 247]. It is predicted that these CYP163 enzymes will also utilize PCP-bound substrates as scaffolds for their single hydroxylation reactions in a similar way to that described for the triple β-hydroxylase P450sky. The structure of P450sky has been determined in the substrate-free form (PDB 4LOF) with an open structure revealing a large solvent exposed active site cavity, similar to those of other ligand-free P450 structures that were shown to bind substrate-loaded PCPs, e.g., OxyB, OxyC, and BioI [131, 248–250]. P450sky contains an unusual additional M-helix at the C terminus, on the proximal face of the structure and lying perpendicular to the L-helix [251]. It was postulated that this extra helix may be involved in redox partner or NRPS machinery interaction(s) [236]. Recently, the structure of P450sky was determined in complex with a PCP protein linked to an azole inhibitor compound (*S*-[2-({N-[(2*R*)-2-hydroxy-3, 3-dimethyl-4-(phosphonooxy)butanoyl]-*beta*-alanyl}amino) ethyl] 1H-imidazole-4-carbothioate) that trapped the otherwise transient interaction between the two proteins, enabling the determination of their structures (PDB 4PWV and 4PXH) [252]. Interestingly, the P450sky–PCP complex occupies a distinct orientation with the carrier protein-bound

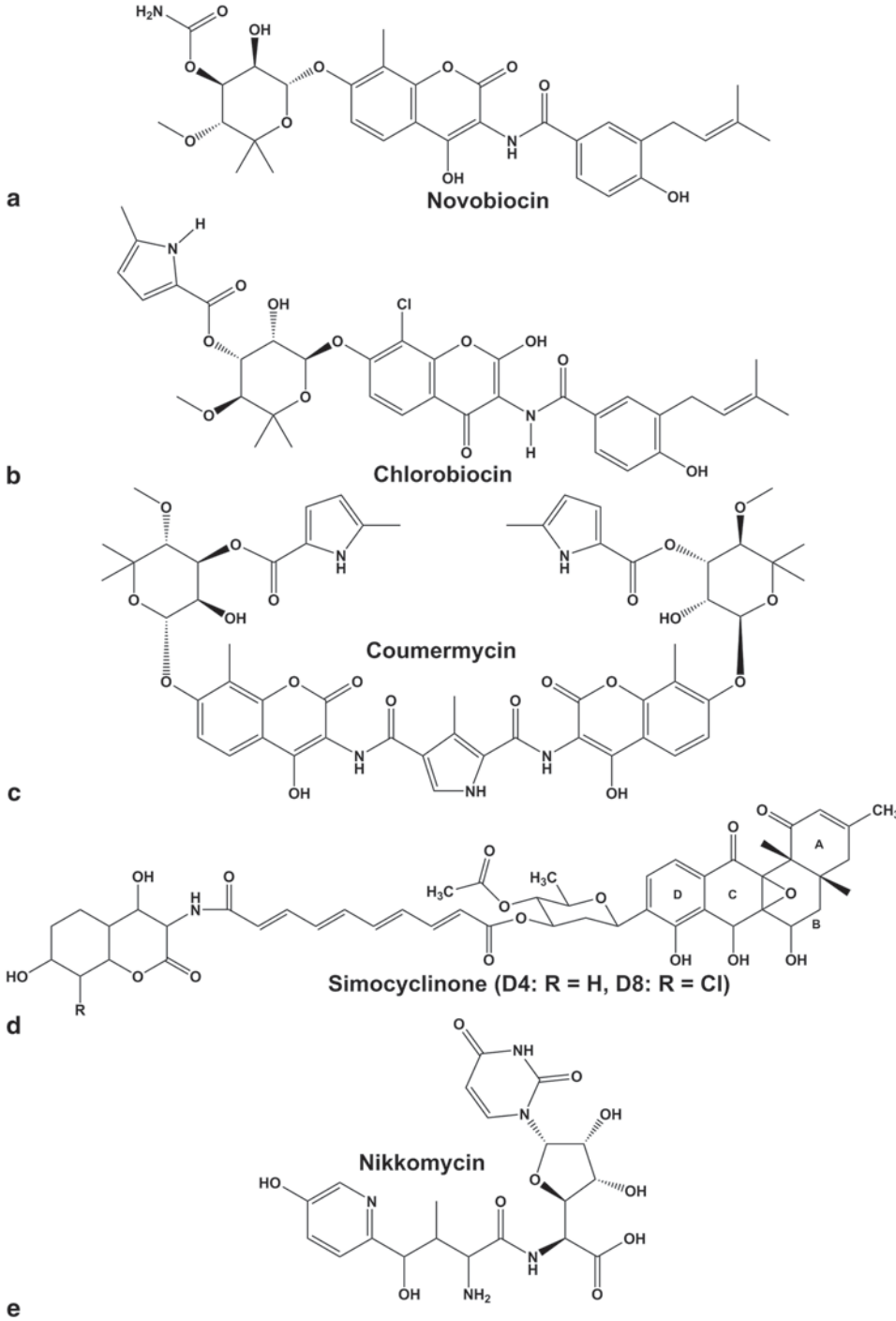

Fig. 6.13 Structure of antibiotics that are generated using the β-hydroxylase CYP162 and CYP163 P450 enzymes. **a** The aminocoumarin novobiocin, the synthesis of which involves the P450 NovI (CYP163A1). NovI is a β-hydroxylase from *S. spheroides* that was shown to generate (2*S*,3*R*)-β-OH-tyrosine from amino acyl-bound tyrosine during novobiocin biosynthesis [243]. **b** The related chlorobiocin, requiring CloI (CYP163A2) [244]. **c** Coumermycin, requiring CumC (CYP163A) [245]. **d** Simoclinone—with the rings of its steroid-like moiety labeled, requiring SimI (CYP163A3) [246, 247]. **e** Nikkomycin—involving NikQ (CYP162A1), a histidine β-hydroxylase from *S. tendae* Tu901, in a similar biosynthetic pathway to NovI [336, 799]

azole ligand situated in different entry channel in comparison to the BioI–ACP complex, the other P450 that has been structurally characterized in complex with a fatty acid substrate-bound ACP. Despite the similarities between the two carrier protein domains, they interact with different regions on their respective P450s. The PCP domain sits above the P450sky G-helix and the PCP α2 and α3 helices form a cleft to accommodate the P450sky G-helix. The P450sky–PCP interface consists of clusters of amino acid residues that form hydrophobic interactions between secondary structural elements from both protein domains. The P450sky M-helix is situated on the other side of the protein structure and is not involved in interactions with the PCP complex. The PCP-linked azole inhibitor is oriented in the substrate access channel in the FG region. In contrast to the P450sky–PCP conformation, the Biol–ACP complex shows the ACP to be located between the BioI B_2 helix and FG helices with interactions with the β1 sheet and the loop between the B and B_2 helices [252]. The ACP-bound fatty acid is situated in a channel between the α-helical and β-sheet domains with the substrate projecting up towards the F-helix to orient the ligand for the BioI-derived oxidative C–C bond cleavage [131] (see the section 'Nonredox partner proteins for microbial P450s in the Redox partner systems and their diversity in microbes'). These structural differences likely reflect the differing P450sky and BioI molecular selectivities and divergent roles in carrier protein-assisted natural product biosynthesis. The unusual multi-hydroxylation reactivity of P450sky with different PCP-linked amino acid substrates during skyllamycin biosynthesis is possibly a result of a carrier protein-derived specificity. It may be the case that the separate amino acid loaded PCP proteins can facilitate different binding conformations on interacting with P450sky, so enabling the three different hydroxylation reactions to enable the progression of the production of skyllamycin.

CYP245A1 (StaP) from *Streptomyces* sp. TR-A0274 catalyzes the aryl–aryl coupling of chromopyrrolic acid in staurosporine biosynthesis [154, 253, 254]. Staurosporine and the structurally related compounds rebeccamycin (Fig. 6.14) [255] and AT2433 [256] (from *Lechevalieria aerocolonigenes and Actinomadura melliaura,* respectively) are indolocarbazole alkaloid antitumor agents (possessing neuroprotective properties) with their activities due to the inhibition of protein kinase or DNA topoisomerase enzymes [257–259]. The CYP245 family is found only in soil and marine dwelling actinobacteria that produce staurosporine-related alkaloid indolocarbazole derivatives [260, 261], with BLAST searches revealing closely related orthologs in *S. longisporoflavus* and *S. purpureus,* along with a large number of relatives in *Salinispora* spp., particularly the subspecies *Salinispora arenicola*. Staurosporine and other indolocarbazole derivatives have an indole (2,3a) carbazole structural core that is linked to a sugar moiety via a C–N bond, with a double deoxysugar linkage specific to staurosporine [262–265]. The StaP substrate, chromopyrrolic acid, is generated via the condensation of two molecules of an indole-3-pyruvic acid imine derived from *L*-tryptophan, and is subsequently converted to staurosporine via a series of enzymatic processes [254, 266, 267]. StaP catalyzes the C5 aryl–aryl coupling of the indole rings of chromopyrrolic acid (Fig. 6.14) [154], likely through a mechanism utilizing compound II as described above for CYP158A2, with a similar StaP tyrosine residue (Tyr351) adjacent to the cysteine thiolate ligand and predicted to provide the reducing equivalent to generate compound II [200]. StaP was originally thought to perform a second oxidative decarboxylation of the biaryl-coupled product to make aglycone derivatives with oxidation(s) on the pyrrole ring [154, 268, 269]. However, it has been determined that these aglycone products are derived both nonenzymatically from the StaP intermediate product [268], as well as being produced by the flavin monooxygenase StaC [265, 267, 270]. The StaC enzyme is responsible primarily for conversion of the biaryl-coupled indolocarbazole to the correct staurosporine-specific aglycone, with a C2 carbonyl group as the sole substituent on the pyrrole ring [265, 270], and prior to the subsequent glycosylation and methylation steps to produce staurosporine. The structure of StaP has been determined in both the ligand-free (PDB 2Z3T)

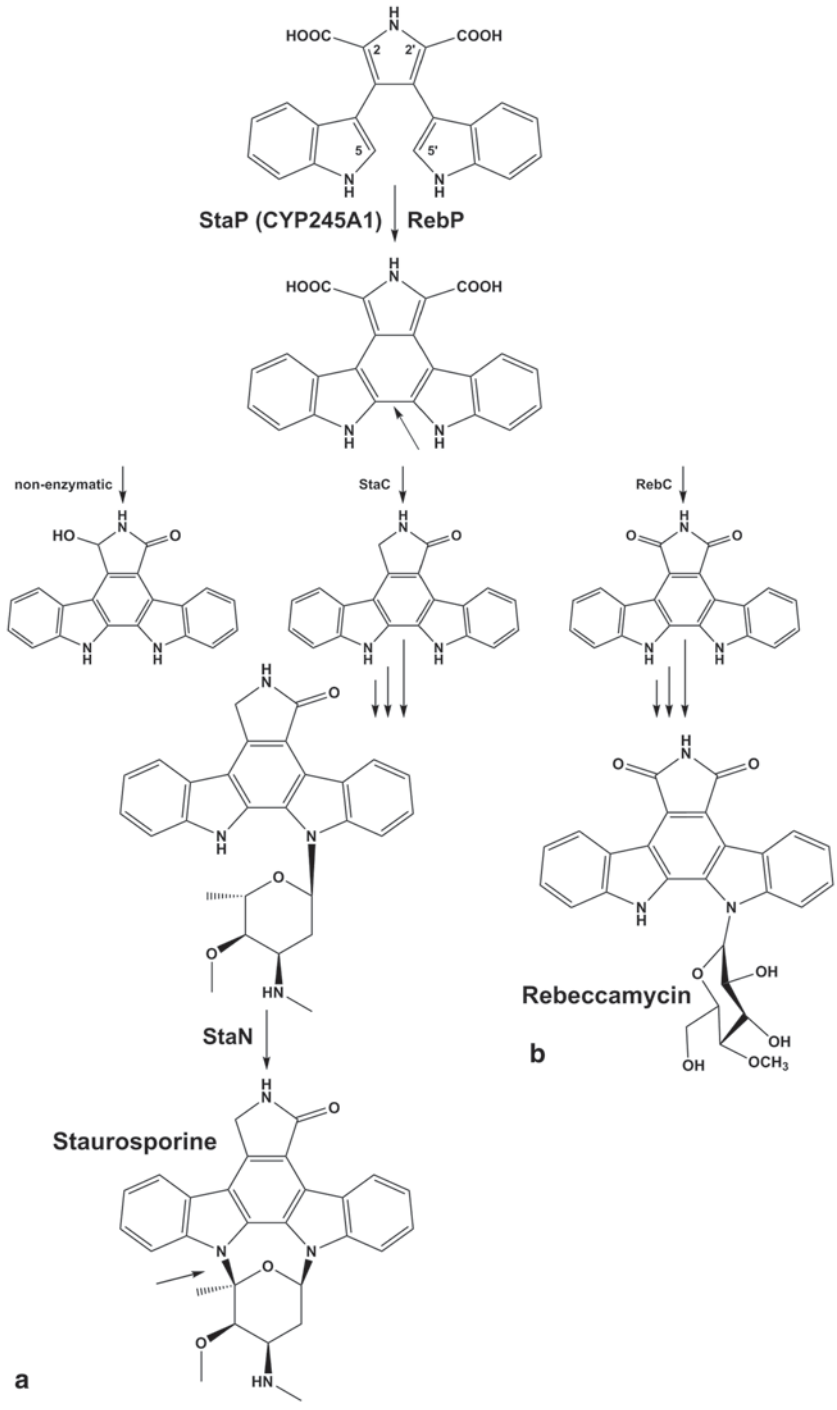

Fig. 6.14 Biosynthesis of staurosporine and the related antitumor drug rebeccamycin. The P450s StaP (CYP245A1) and RebP (CYP245A2) catalyze the C5 biaryl coupling of chromopyrrolic acid (*arrow*) in the respective pathways [154, 269]. The products are further converted by the flavin monooxygenases StaC and RebC, respectively, to the decarboxylated and oxidized intermediate forms [268, 269]. The P450 StaN (CYP244A1) catalyzes one of the final steps in the biosynthetic pathway, forming a C–N bond between the indolocarbazole and deoxysugar moieties (*arrow*) [266, 802]. This reaction occurs prior to methylation reactions that lead finally to the production of staurosporine (**a**). The synthesis of rebeccamycin (**b**) does not involve the C–N bond formation and an equivalent P450 to StaN does not occur in this pathway [850]

and substrate-bound (PDB 2Z3U) forms [154], with open/closed conformations similar to those described for other P450s (e.g., BM3 and CYP119A1) [92, 271]. The substrate-bound structure reveals three molecules of chromopyrrolic acid, with one clearly in the active site cavity, a second located external to the active site between helix B′1 and sheet β-1 and the third between β-3,1 and helix B of another StaP molecule within the asymmetric unit of the crystal lattice. The significance of these secondary binding sites is not clear and they may be artifactual, although the region encompassing helix B′1 and sheet β-1 is extremely flexible and has been proposed as a substrate entry site for several P450s, and thus may be the route of entry/exit for chromopyrrolic acid [154]. The active site-bound chromopyrrolic acid is well defined in an apparent 'twisted butterfly' conformation, with the indole rings held in place by π–π interactions and a number of hydrogen bond interactions between the rest of the substrate molecule and StaP amino acid residues. For the biaryl-coupled catalysis to occur resulting in ring closure, the chromopyrrolic acid substrate would have to move within the active site cavity to be closer to the reactive heme iron–oxo (likely compound II) species. Molecular modeling coupled with mutagenesis studies have predicted that, in the absence of a different substrate-binding mode, the StaP mechanism likely involves proton-coupled electron transfer (potentially involving active site histidine residue(s), e.g., His250), assisted by an essential active site water dyad [272]. Further experimental evidence may be required to elucidate the full StaP reaction mechanism, but many active site residues, including His250, are conserved in the related indolocarbazole rebeccamycin-producing RebP (CYP245A2) and AT2433-producing AtmP (CYP245A3) biosynthetic P450s [273]. In addition to StaP, a second putative P450 enzyme (StaN, CYP244A1) is believed to be involved in one of the latter steps of staurosporine biosynthesis, prior to the final StaM methyltransferase reactions. StaN appears to catalyze the second C–N linkage between the aglycone and the deoxysugar moieties to form the intermediate *O*-demethyl-*N*-demethyl-staurosporine (Fig. 6.14), as determined by gene disruption studies in vivo [266, 274]. There are no orthologous StaN P450s detected in *L. aerocolonigenes* and *A. melliaura,* where the second deoxysugar linkage is absent in the rebeccamycin and AT2433 derivatives [273]. StaN is thus a staurosporine-specific P450 and, although not yet fully characterized, StaN likely represents an unusual example of a C–N bond forming P450 enzyme.

TxtE (CYP1048A1) is an intriguing P450 enzyme with a novel catalytic role in the biosynthesis of a cyclic dipeptide phytotoxin. It is found in *S. scabiei* 87.22 and in other plant-pathogenic *Streptomyces* species, e.g., *S. ipomoeae, S. turgidiscabies,* and *S. acidiscabies* [275–279]. In contrast to the primarily antibiotic biosynthetic *Streptomyces* P450s, TxtE is involved in the production of the plant toxin thaxtomin, responsible for potato common scab [280, 281]. Thaxtomins have the core structure of cyclo-(*L*-4-nitrotryptophyl-*L*-phenylalanyl) [282] with 11 different types of thaxtomins isolated and characterized that differ only in *N*-methyl and hydroxyl substituent groups [283]. Thaxtomin A is the dominant form and is a key virulence factor in the *Streptomyces* spp. pathogenicity (Fig. 6.15) [275]. The biosynthetic pathway of thaxtomin A contains five genes that lie on a pathogenicity island [284] and is encoded by two NRPSs (TxtA and TxtB), a nitric oxide synthase (TxtD), and two P450s (TxtC and TxtE; Fig. 6.15) [107, 275, 284]. TxtE is the pivotal enzyme in thaxtomin A biosynthesis catalyzing the direct regio-specific (C4) nitration of the indole ring of *L*-tryptophan, utilizing nitric oxide (NO) produced by the genetically adjacent nitric oxide synthase (TxtD) in the presence of O_2, redox partners, and NADPH to produce *L*-4-nitrotryptophan (Fig. 6.15) [107]. The unusual nitration action of TxtE would require a nitrating species, thus deviating from the conventional P450 catalytic cycle (e.g., [279, 285]; Fig. 6.4). It was proposed that TxtE forms a ferric superoxy complex that reduces NO to give rise to a ferric peroxynitrite species, which then yields NO_2 and compound II via homolytic cleavage. *L*-tryptophan nitration could then proceed by addition of NO_2 and compound II-mediated hydrogen atom extraction (or *vice versa*), resulting in an

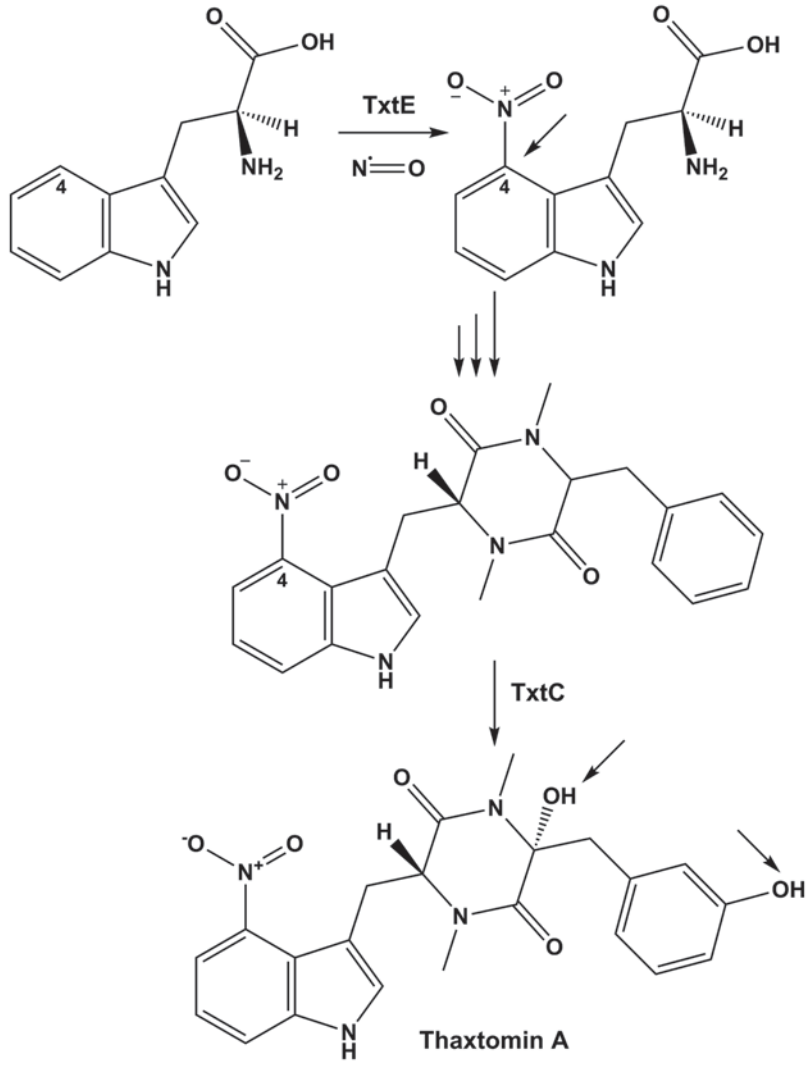

Fig. 6.15 The role of TxtE in synthesis of the phytotoxin thaxtomin. The P450 TxtE (CYP1048A1) catalyzes the C4 nitration of *L*-tryptophan to produce 4-nitrotryptophan (indicated by an *arrow*). The NO required is generated by a nitric oxide synthase (NOS) enzyme (TxtD) encoded adjacent to *txtE* on the *S. turgidiscabies* genome. 4-nitrotryptophan undergoes a condensation reaction with *L*-phenylalanine catalyzed by the enzymes TxtA/B (not shown) to produce the *N*,*N*′-methyldiketopiperazine [107, 279]. The P450 TxtC (CYP246A1) then catalyzes two further hydroxylations on the diketopiperazine and the phenylalanine moieties (shown by *arrows*) to produce thaxtomin A [107, 275]

Fe(III)–OH species. Alternatively, nitration may occur by classical electrophilic aromatic substitution following protonation-triggered heterolytic cleavage of the ferric peroxynitrite species to produce NO_2 and an Fe(III)–OH species. The resting state of the enzyme (Fe(III)–OH_2) is regenerated by protonation of the Fe(III)–OH species, regardless of the mechanism of nitration [107]. The characterization of TxtE therefore reveals an unprecedented nitration in a biosynthetic pathway, and a new activity for a P450 enzyme (where a nitro group would typically be derived from oxidation of an amine) [278, 279]. The thaxtomin A diketopiperazine core (cyclo-(*L*-4-nitrotryptophyl-*L*-phenylalanyl)) is produced by the condensation of TxtE-derived *L*-4-nitrotryptophan

and *L*-phenylalanine catalyzed by TxtAB to produce N, N′-dimethyldiketopiperazine [275, 286]. The thaxtomin core is tailored by the P450 TxtC that catalyzes the hydroxylation of both the diketopiperazine moiety and the phenyl group to produce thaxtomin A (Fig. 6.15) [107, 275]. Further characterization of TxtC, including structural analysis, should reveal the mechanism by which it achieves hydroxylation at two chemically different and spatially distinct sites. The structure of the nitrating P450 TxtE (PDB 4L36) revealed the presence of two imidazole molecules, with one coordinating directly to the heme iron and the second interacting simultaneously with two glutamate (Glu187) residues from two TxtE molecules within the asymmetric unit. The overall TxtE structure shows an additional loop situated between the two B′ helices (B′1 and B′2) and disorder in the FG loop, presumably reflecting the flexibility of this region [278]. A defined solvent-filled channel, likely involved in substrate access, is seen between the B′1 and G helices, similar to other P450 enzymes [112, 229]. There is also a kink in the I-helix close to the conserved threonine (Thr250) that reveals a putative proton pathway, with a continuous network of water molecules leading from the active site to the outside of the protein. Substrate docking and subsequent mutagenesis experiments highlight active site amino acids that have roles in substrate recognition and binding [278]. Further studies to characterize structures of substrate-bound complexes will assist in understanding the nitration mechanism. Recent reports have revealed that synthetic stereoisomers of thaxtomin A exhibit a range of phytotoxic, fungicidal and antiviral activities [287]. TxtE is thus an interesting *Streptomyces* P450 in both reactivity and mechanism, and is a good candidate for future use in biotechnological applications, with diversification of its activity towards thaxtomin A analogs possibly giving rise to novel antibiotic products.

6.2.3.2 Other Biosynthetic Actinomycete P450s

Beyond the *Streptomyces,* there are a number of structurally characterized actinomycete P450s from distinct P450 families with diverse functions, many of which are involved in the generation of different natural products (Table 6.3). CalE10 (CYP105W1) and CalO2 (CYP248A1) from *Micromonospora echinospora* are distinct P450s involved in the biosynthesis of calicheamicin (Fig. 6.16) [230, 288]. Calicheamicin is a ten-membered nonchromoprotein enediyne that, unlike the nine-membered enediyne counterparts, does not require a subsidiary protein (chromoprotein) for stability [289]. Calicheamicin is an extremely potent cytotoxic agent with antimicrobial properties, which docks in the minor groove of target DNA/RNA, causing lethal oxidative strand scission [290–292]. The structure of calicheamicin consists of an aryltetrasaccharide, composed of four monosaccharide units and one hexa-substituted benzene (orsellinic acid) moiety, and a core aglycone bicyclo[7.3.1]tridecadiynene with an allylic trisulfide side group [291, 293]. The aryltetrasaccharide hydroxylamino glycosidic bond is responsible for locating and binding the enediyne drug in the minor groove of DNA, forming hydrophobic interactions with a small T/C-rich region within the DNA helix. The aglycone part of the enediyne acts as the ‘warhead’ and is activated via nucleation of the trisulfide, which undergoes cycloaromatization with the aglycone core and produces the highly reactive diradical 1,4-didehydrobenzene. This diradical subsequently abstracts hydrogen atoms from the deoxyribose backbone of DNA, ultimately leading to strand scission and destruction of tumor or cancer cells [290–295]. However, the extremely high reactivity/potency of calicheamicin is so great that issues with lack of specificity for tumors present major toxicity issues. This problem was solved by the attachment of calicheamicin to tumor- and other desired target-specific monoclonal antibodies with clinical success (e.g., [296, 297]). The biosynthesis of the enediyne core of calicheamicin and other similar compounds (both nine- and ten-membered) involve a common enediyne polyketide synthase (named PKSE) [295, 298]. The calicheamicin-specific orsellinic acid moiety and other substituent groups are produced via unique iterative type I polyketide synthases, with at least 20 genes in the calicheamicin biosynthetic gene cluster including those for the two

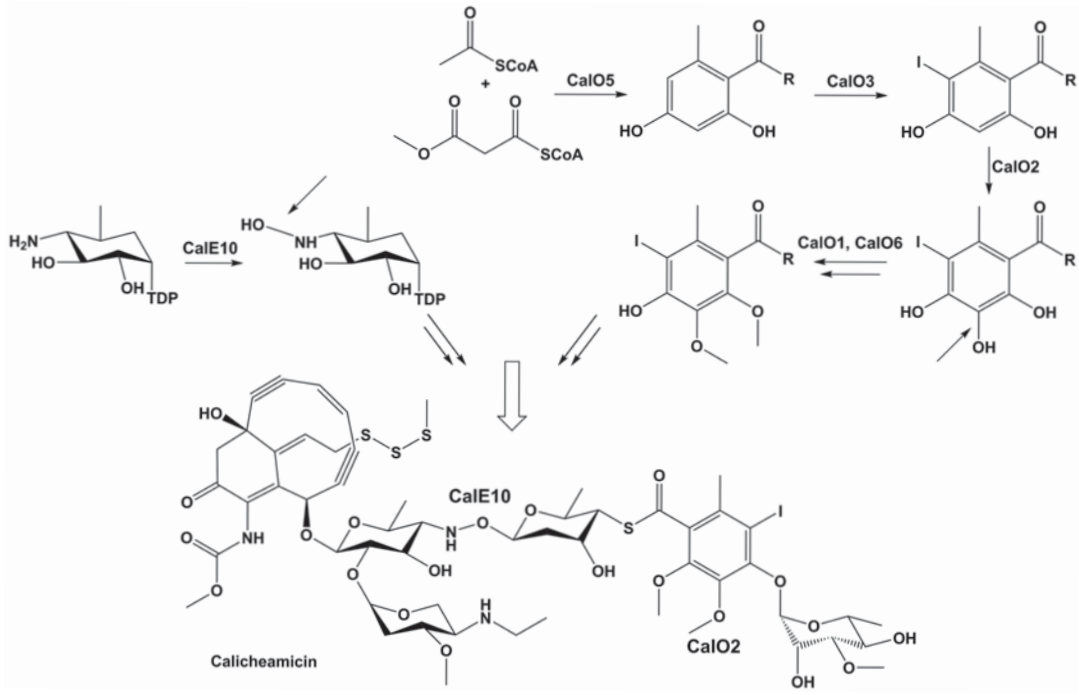

Fig. 6.16 The calicheamicin biosynthetic pathway. The scheme shows the CalO2-catalyzed hydroxylation (shown by an *arrow*) of the iodinated orsellinic acid moiety, with R predicted to be an ACP or CoA thioester [230]. CalE10 is a regio-specific NDP-amino sugar N-oxidase involved in the production of the 4-hydroxyamino-6-deoxy-α-D-glucose moiety (*arrow*) prior to glycosyl transfer in calicheamicin biosynthesis [288]. The final structure of calicheamicin is shown at the bottom of the panel, with the position of the P450-derived oxidations indicated by the enzyme names

P450 enzymes. CalE10 is a regio-specific TDP-*alpha-D*-4-amino-4,6-deoxyglucose *N*- oxygenase involved in the formation of the calicheamicin hydroxylamino glycoside, an unusual naturally occurring N-oxidized amino sugar (Fig. 6.16) [288]. The genomic locations of CalE10 and CalO2 are distinct, with CalO2 clustered with other genes involved in orsellinic acid synthesis. The CalE10 gene instead lies adjacent to a gene implicated in sugar biosynthesis and to several other genes of uncertain function [230, 295]. CalE10 is similar to a number of uncharacterized CYP105 enzymes from *Salinospora* spp., *Actinoplanes* spp., and *Amycolatopsis* spp. [19], including the *A. orientalis* epothilone B hydroxylase that produces epothilone F [299]. CalO2 is a distinct P450 with only a handful of orthologs identified in *Salinispora arenicola* CNS-205. In contrast to CalE10, CalO2 is located within a biosynthetic subcluster for the aromatic moiety of the calicheamicin aryltetrasaccharide portion, containing CalO1 (an AdoMet-dependent orsellinic acid *O*-methyltransferase) [300], CalO3 (a flavin-dependent halogenase), CalO4 (a 3-oxoacyl-ACP synthase III), CalO5 (an orsellinic acid synthase and type I PKS), and CalO6 (an AdoMet-dependent orsellinic acid C2 O-methyltransferase) [230]. CalO2 is involved in the hydroxylation of the aromatic ring of iodo-substituted orsellinic acid (Fig. 6.16) with a likely preference for ACP or coenzyme A (coA)-bound substrates, indicated by a higher affinity for substrates with an *N*-acetylcysteamine group (a model carrier protein linker) than for free aromatic acids. The preferred presence of iodine also highlighted that the halogenase CalO3 reaction likely precedes that of CalO2 in the biosynthetic pathway. The structure of CalO2 was solved in the ligand-free form (PDB 3BUJ), revealing an interesting additional two-helix bundle encompassing the B′ and B″ he-

lices [230], similar to that described for CYP151 (AurH) from *S. thioluteus* [226] (Fig. 6.11). This two-helix bundle closes over the active site and blocks solvent channels, but maintains a central, open cavity with the potential for substrate access. Docking studies involving CalO2 and the orsellinic acid synthase (CalO5) ACP domain suggest a well-fitted interaction between the CalO2 B′ helix and the ACP helix 2 and reveal a plausible binding mode for the ACP-bound substrate [230]. The pimelic acid synthase BioI (CYP107H1) from *B. subtilis* is the first example of a P450/ACP–fatty acid complex and provided the paradigm for a new P450 mechanism that utilizes the accessory carrier protein to regulate substrate specificity, whilst providing a scaffold for the oxidative reaction [131]. BioI is described in more detail in the section 'Nonredox partner proteins for microbial P450s' of *Redox partner systems and their diversity in microbes*.

The antibiotic mycinamicin II produced by *Micromonospora griseorubida* A11725 is a member of the 16-membered macrolide mycinamicins with potent activity against Gram-positive bacteria and mycoplasma, including some drug-resistant pathogenic bacteria such as *Legionella* spp. [301–303]. The structure of mycinamicin is composed of a central macrolactone with O-linked dimethylated desosamine and mycosine 6-deoxyhexose sugar substituents at the C21 and C5 positions, respectively [302]. The mycinamicin biosynthetic gene cluster contains the two P450s MycCI (CYP105L2) and MycG (CYP107E1) that are located on either side of a central metal-dependent *S*-adenosyl-*L*-methionine methyltransferase [304]. MycCI catalyzes the C21 methyl hydroxylation of mycinamicin VIII (Fig. 6.17), the earliest glycosylated form of the macrolide in the biosynthetic pathway. The activity of MycCI is dependent on the adjacent ferredoxin MycCII [305, 306]. The C21 hydroxylation is the target for 6-deoxyallose addition by the glycosyltransferase MycD, forming mycinamicin VII [304]. MycCI is similar to a number of methyl hydroxylase antibiotic biosynthetic P450s that are members of the CYP105 family, including TylHI involved in tylosin production in *S. fradiae* (Fig. 6.18a) [307] and ChmHI involved in chalcomycin production in *S. bikiniensis* (Fig. 6.18b) [308] that are also adjacent to FDs. MycG is an interesting multifunctional P450 that catalyzes the C14 hydroxylation and successive C12–C13 epoxidation of the macrolactone ring of mycinamicin during the final tailoring stages to produce mycinamicin II (Fig. 6.17) [305]. Surprisingly, the MycCI ferredoxin (MycCII) does not support the activity of MycG and further (as yet uncharacterized) redox partners are likely utilized in reactions involving MycG [306]. Other dual function P450s that perform similar sequential reactions include the aureothin synthase AurH (CYP248A1) from *S. thioluteus,* discussed above (Figs. 6.10b and 6.11) [226], and the tirandamycin synthase TamI from *Streptomyces* sp. 307–9 that catalyzes three successive hydroxylations and a single epoxidation reaction on the bicyclic ketal component of the natural product tirandamycin C (Fig. 6.18c) [309]. The P450 GfsF from *S. graminofaciens* is involved in the biosynthesis of the 16-membered macrolide antibiotic FD-891 with cytotoxic properties (Fig. 6.18d). GfsF catalyzes the sequential epoxidation and hydroxylation reactions on adjacent carbons to produce FD-891, but performs these reactions in the reverse order compared to those for MycG [310]. The crystal structure of MycG has been solved in the ligand-free (PBD 2YGX) and substrate-bound forms with the native substrates mycinamicin IV and V (e.g., PDB 2Y46 and 2Y5N) [311]. The structures of MycG reveal a relatively open conformation with a large active site cavity and a short FG loop. Few differences are observed between the substrate-free and -bound conformations, or between structures determined from different crystal space groups. The ligand-bound structures have the mycinamicin IV and V substrates in very similar orientations—bound orthogonal to the heme plane. The dimethoxylated mycinose sugar moieties are bound within the active site cavity and form hydrophobic interactions with the heme macrocycle and active site amino acids. The desosamine groups of the second sugar extend out of the active site towards the surface of the protein and interact with the

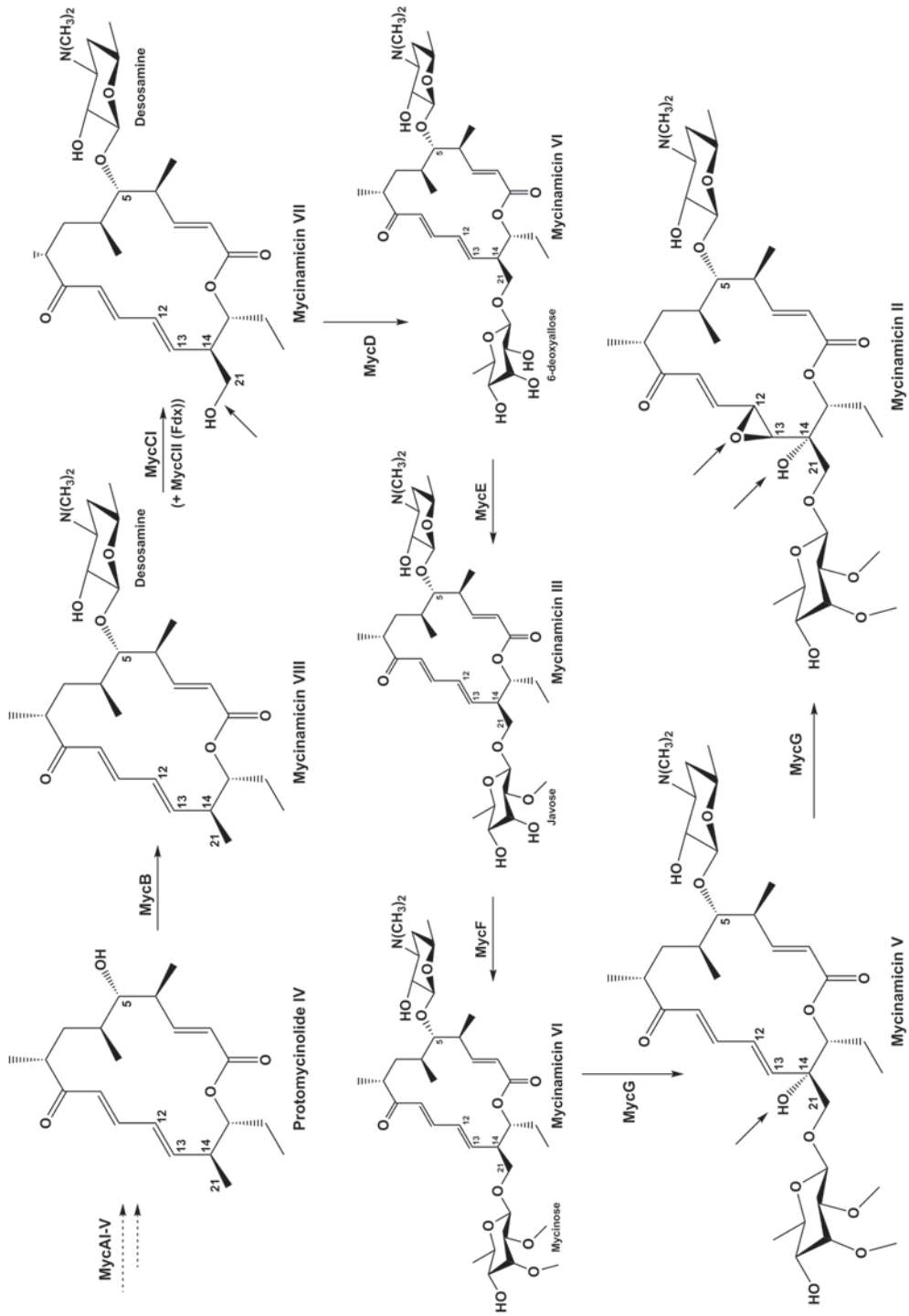

Fig. 6.17 The mycinamicin biosynthetic pathway. The P450 MycCI (CYP105L2) catalyzes the C21 hydroxylation of mycinamicin (MC) VIII, the earliest glycosylated (desosamine) form of mycinamicin derived from protomycinolide IV. The MycCI C21 hydroxylation produces MCVII (position of hydroxylation indicated by an *arrow*) and the activity of MycCI is dependent on its chromosomally adjacent ferredoxin (Fdx) MycCII. MCVII undergoes glycosylation and methylation reactions catalyzed by MycD–MycF to produce MCIV, the MycG substrate. The P450 MycG (CYP107E1) performs a C14 hydroxylation to produce MCV, and then a C12–C13 epoxidation reaction (*arrows*) to generate the final antibiotic mycinamicin II [305, 306, 311]

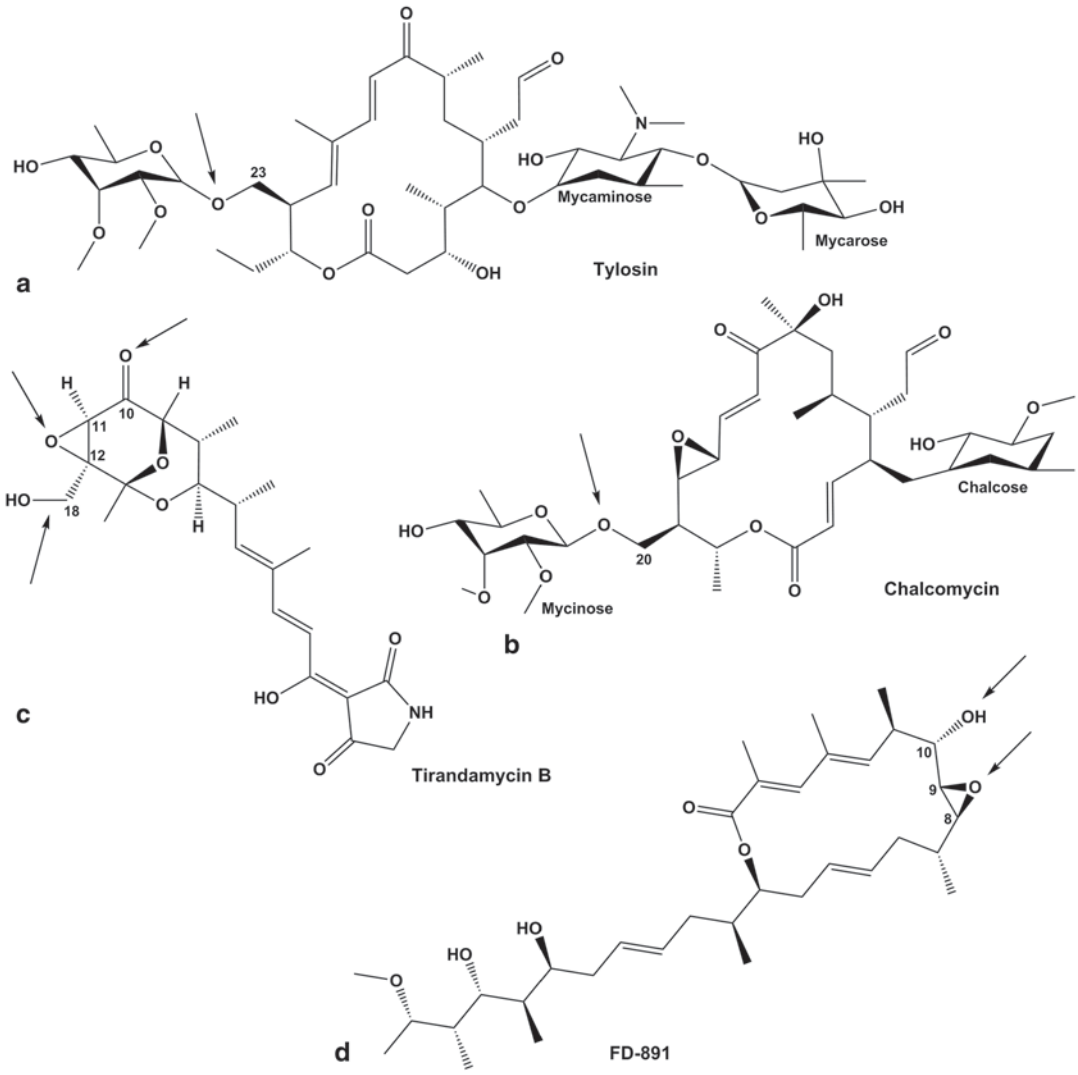

Fig. 6.18 Antibiotic compounds involving P450-dependent synthetic reactions related to those catalyzed by the mycinamicin biosynthetic P450s. Reactions shown in **a** and **b** involve similar hydroxylations to those done by MycCI. Reactions in **c** and **d** are successive oxidations similar to those done by MygG. P450-mediated oxidations are highlighted with *arrows* in each case. **a** Tylosin—TylHI (CYP105L1) from *S. fradiae* catalyzes the C23 methyl macrolide hydroxylation [804, 805]. **b** Chalcomycin—ChmH1 from *S. bikiniensis* catalyzes the C20 methyl macrolide hydroxylation [308]. **c** Tirandamycin B—TamI from *Streptomyces* sp. 307-9 catalyzes the C10 oxidation of tirandamycin **c**–**e**, and then the C11–C12 epoxidation and C18 hydroxylation to form tirandamycin B [309, 806]. **d** FD-891—GfsF from *S. graminofaciens* catalyzes the C8–C9 macrolide epoxidation and then the C10 hydroxylation (the opposite order to reactions done by MycG) to produce FD-891 [310, 797]

FG loop. It is proposed that the methoxy groups of the mycinose mediate substrate recognition by MycG and play a role in discrimination between closely related substrates, thus ensuring that the correct catalytic function can occur at the distinct sites of mycinamicin IV and V. However, these substrate orientations observed in different MycG structures are unlikely to be in the correct

position for oxidative catalysis, with the heme iron distal water retained and a large distance (9–10 Å) between the C14 and C12–C13 positions of the macrolactone rings and the putative active species at the heme iron. NMR relaxation and modeling studies suggest that mycinamicin IV may penetrate further into the active site to allow a catalytically productive orientation and to enable C14 hydroxylation to yield mycinamicin V. However, it is unclear how the epoxidation of mycinamicin V would occur across the C12–C13 double bond from these studies. It is thus predicted from the structural data that the mycinamicin substrates are bound to MycG in an orientation that precedes the catalytically relevant mode, with the mycinose moieties conferring substrate recognition and specificity prior to substrate reorientation and catalysis [311].

An interesting group of biosynthetic P450s is the Oxy enzymes that mainly constitute the CYP165 family from various *Amycolatopsis* spp. These are involved in the biosynthesis of glycopeptide antibiotics, including vancomycin (*A. orientalis*; Fig. 6.19a) [312], balhimycin (*A. balhimycina*; Fig. 6.19a) [313], and teicoplanin (*A. teichomyceticus*; Fig. 6.19b) [314]. The glycopeptide antibiotics are used in the treatment of Gram-positive bacterial infections that are resistant to other classes of antibiotics, such as MRSA [315]. They are inhibitors of bacterial cell wall peptidoglycan synthesis and act by binding to the dipeptide terminus *D*-Ala–*D*-Ala of peptidoglycan precursors, preventing the transpeptidation and transglycosylation reactions essential for peptidoglycan cross-linking [289, 316]. The CYP165 family has multiple members that are restricted to certain *Streptomyces* and *Actinoplanes* spp. in addition to *Amycolatopsis* spp. [165]. The Oxy P450s were shown to catalyze oxidative coupling reactions with single PCP-bound substrates (e.g., [237–240]). OxyA (CYP165A3), OxyB (CYP165B3), and OxyC (CYP165C4) in vancomycin/balhimycin synthesis, along with OxyE (CYP165C3) in teicoplanin synthesis, are responsible for catalyzing the cross-linking of PCP-loaded aromatic amino acid side chains in the glycopeptide antibiotic aglycone core [251, 317]. OxyB performs the first oxidative coupling between phenol rings C and D, forming an aryl–ether bridge between the side chains of residues four and six (Fig. 6.19) [248, 318–320]. The second cross-link is performed by OxyA, which catalyzes a further aryl–ether bridge formation reaction between the D and E phenol rings of the side chains of residues two and four (Fig. 6.19) [321–323]. The OxyB- and OxyA-derived diarylethers are formed by coupling of separate 3-chloro-β-hydroxytyrosine and 4-hydroxyphenylglycine residues. In teicoplanin biosynthesis, the oxidative phenolic coupling of rings F and G between the side chains of residues one and three precedes the second Oxy A-catalyzed step. This additional phenolic cross-link is unique to the teicoplanin-type antibiotics and is catalyzed by OxyE (Fig. 6.19b) [239, 240, 324]. The final biaryl cyclization of rings A and B is formed by 3,5-dihydroxyphenylglycine and 4-hydroxyphenylglycine of residues five and seven and is catalyzed by OxyC (Fig. 6.19) [249, 321]. The structures of OxyB (PDB 1LFK, 1LG9, 1LGF) [248], OxyC (PDB 1UED) [249], and OxyE (PDB 3O1A and 3OO3) [239, 240] have been solved in the ligand-free forms. OxyB and OxyC display high levels of structural similarity and reveal open conformations, with the FG helices rotated out of the active site to leave a large open cavity that is likely to enable binding of the bulky PCP-bound substrates [248, 249, 317, 325, 326]. They also possess a common additional N-terminal β hairpin (β0) that appears to have a role in the stabilization of the active site cavity through the formation of hydrogen-bonding networks initiated by a β0 arginine residue [248, 249]. OxyC has an additional β strand (β10) and A″ helix at the N-terminus, as well as a C-terminal M-helix that are not present in OxyB or OxyE [239, 240, 248, 249]. OxyB, OxyC, and OxyE display structural similarities to the nitric oxide reductase P450nor (CYP55A1) [327] (see the section ‘P450 systems that bypass redox partners’ in the *Redox partner systems and their diversity in microbes*), although differ primarily in the orientation of the FG and B′ helices, with the open OxyB/C and OxyE active site cavities re-

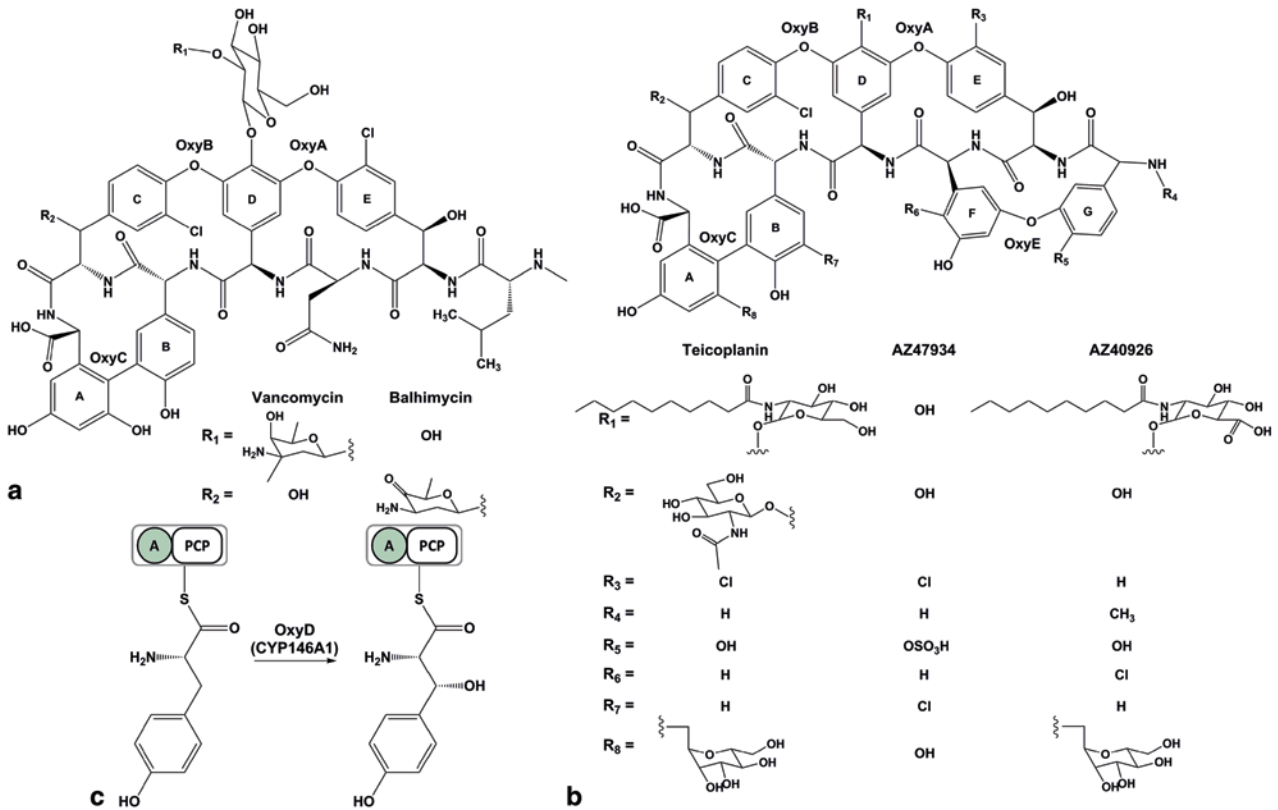

Fig. 6.19 Vancomycin- and teicoplanin-type antibiotics and the action of the Oxy P450s in *Amycolatopsis* spp. The antibiotic precursors are bound to peptidyl carrier proteins (PCPs) during the production process [251]. **a** Vancomycin and balhimycin, showing their respective substituent groups and the P450-mediated PCP-bound products. OxyB (CYP165B) [318–320] and OxyA (CYP165C) [321–323] catalyze the oxidative phenol coupling of the C–D and D–E rings, followed by the biaryl coupling of rings A and B by OxyC (CYP165C) [249, 321]. **b** Teicoplanin and the related antibiotics A47934 and A40926. In addition to the other Oxy enzymes, OxyE (CYP165D) performs the oxidative phenol coupling of the additional F–G rings as the second step following the action of OxyB in teicoplanin biosynthesis [239, 240, 324]. **c** The formation of *L*-3-(*R*)-hydroxytyrosine catalyzed by OxyD (CYP146A1 in *A. mediterranei*) that is required for vancomycin and teicoplanin biosynthesis. The tyrosine substrate is loaded on an NPRS containing the A-adenylation domain and the PCP-peptidyl carrier protein domain. The hydroxytyrosine precursor product is cleaved from the NRPS through the action of an adjacent thioesterase. NPRS nonribosomal peptide synthetase [237, 238]

flecting their larger substrates. The central portions of the I-helix in all three Oxy structures display a small kink, common in P450 structures and predicted to be involved in oxygen binding and proton delivery [24]. In the structure of OxyB, the conserved threonine is replaced by an asparagine residue (Asn240) with its side chain pointing into the active site to form a hydrogen bond with the heme axial water, and to create a more pronounced kink than is observed for OxyC and OxyE in the I-helix that allows for an additional OxyB water molecule [248]. OxyC retains the conserved threonine (Thr249) and the preceding acidic glutamate (Glu248) with Thr249 hydrogen bonding to the carbonyl oxygen atom of the active site glycine (Gly245) [249]. The structure of the teicoplanin cross-linking OxyE displays the highest similarity to ligand-free CYP105 family members, followed by OxyB, OxyC, and P450nor [248, 249, 327]. The structure of the teicoplanin cross-linking OxyE displays the highest similarity to ligand-free CYP105 family members [167, 168] followed by OxyB, OxyC [248, 249], and P450nor [327]. Sequence analysis and BLAST searches also reveal OxyA and OxyE to have the highest similarity (46% identity) amongst the Oxy orthologs of teicoplanin-type glycopeptide-producing organisms [239, 240]. Furthermore, OxyE shares a near-identical secondary structure in the putative substrate recognition and binding regions to that predicted for OxyA. This may reflect a similar substrate-binding orientation, as they both perform successive coupling steps during the production of teicoplanin-type antibiotics. In contrast to OxyB/C, the OxyE FG helices are rotated towards the active site, resulting in a more closed conformation. However, the heme is still solvent exposed with the FG helices forming a cap over the I-helix, rather than a lid over the cavity itself [239, 240]. The active site of OxyE extends into an additional pocket located over the β1 sheet and is proposed to facilitate docking to the second residue of the teicoplanin scaffold that is bound to the PCP [240]. A number of hydrogen-bonding interactions are observed between the I-helix and other secondary structural elements (including residues on the F and G helices, the B′C loop, and the CD loop), and these help to stabilize the active site cavity. This active site contains an acidic glutamate residue (Glu229) and has a glutamine (Gln230) in place of the conserved threonine. Although the Gln230 side chain points into the active site cavity, it does not hydrogen bond to any active site water molecules. This suggests it may not participate in protonation of iron–oxo species in the OxyE P450 catalytic cycle. An active site methionine (Met226) occurs in OxyE instead of an alanine or glycine typically observed within an I-helix motif (A/GGXXT) in P450s. This motif contains the conserved threonine replaced by Gln230 in OxyE [24, 327, 328]. Met226 projects across the heme face and forms a hydrogen bond between its side-chain sulfur atom and the heme axial water ligand. This bulky methionine residue is conserved in the OxyE ortholog StaG (CYP165D1) (87% identity) from *S. toyocaensis* [324] and in other uncharacterized orthologs identified in BLAST searches, but not in the Dbv13 (CYP165D2, 73% identity to OxyE) ortholog from *Nonomuraea sp.* 39727 [329]. These orthologous enzymes are predicted to catalyze the analogous cross-linking of aromatic side chains of residue one and three in the production of the teicoplanin-type glycopeptide antibiotics A47934 and A40926 produced in *S. toyocaensis and Nonomuraea sp.* 39727, respectively (Fig. 6.19b). It is unclear whether the OxyE Met226 residue plays an important role in substrate orientation and/or catalysis, or would have to move out of the heme plane to facilitate oxygen binding [239, 240]. An interesting feature of the CYP165 enzymes (particularly OxyB, OxyC, and OxyE) is the apparent substrate specificity derived from elements of both the PCP-bound substrate and, in the latter P450-mediated reactions, whether different substrate molecules have undergone phenol-coupling reactions. For instance, OxyE possesses the ability to select preferentially for substrates that only have the PCP-bound C–O–D phenolic cross-link catalyzed by OxyB [240]. This ensures that the actions of the Oxy enzymes are incorporated correctly during the production of the glycopeptide antibiotics [317, 324, 330, 331]. Furthermore, OxyB and OxyC display the least constraints for

PCP-bound substrate specificity and are perhaps the most likely candidates for engineering modified variants of the aglycone core that may facilitate the production of novel glycopeptide antibiotics [317, 320, 330, 331].

OxyD (CYP146A1) is an important amino acid hydroxylase that catalyzes the formation of *L*-β-*R*-hydroxytyrosine, an essential precursor of the vancomycin-type and teicoplanin-type aglycone core (Fig. 6.19c) [237, 238, 332]. The CYP146 family is unique to the glycopeptide antibiotic-producing strains of *Amycolatopsis* spp. [165] and BLAST searches reveal further uncultured organisms that likely have similar roles in antibiotic production. OxyD is part of a three-gene operon and is cotranscribed with an NRPS (BpsD)-containing single adenylation and PCP domains [333], and a thioesterase (Bhp) [334]. The OxyD substrate tyrosine is loaded onto the PCP domain of the NRPS that defines P450 substrate specificity and serves as a scaffold for the OxyD hydroxylation reaction, β-*R*-hydroxytyrosine is subsequently cleaved from the NRPS by the thioesterase [237]. Similar reactions and gene operons have been described for the unrelated P450s NikQ (CYP162A1) and NovI (CYP163A1) that catalyze the β-hydroxylations of histidine and tyrosine in the biosynthetic pathways of novobiocin [243] and nikkomycin [335, 336] (Fig. 6.13a and e), respectively. The structure of OxyD has been determined in the substrate-free form (PDB 3MGX) with an open conformation [237]. A number of hydrogen bonds between active site residues and secondary structural elements form the open active site cavity. These primarily involve interactions between the FG helices and the I-helix, mediated by hydrogen bonding of the F-helix Asn169 and G-helix His188 with the I-helix Arg241 and Asp230 residues, respectively. The loop portion between the FG helices also interacts with a β strand in the β1 sheet of another OxyD structure in the asymmetric unit, pulling it away from the active site [237]. These interactions thus orient the FG helices to form a cap above the I-helix and impose the open conformation, rather than extending over the heme and forming a lid over the active site cavity, as observed in many P450 structures [24, 112, 229]. It is possible that other structural rearrangements occur upon substrate binding. However, comparisons with other amino acyl-PCP substrate-binding P450s, in both structural and amino acid alignments, reveal regions of similarity in the active site and suggest a common motif involved in the interactions with the PCP domain. Furthermore, the degree of interactions involved in the retention of the open conformation is suggestive that OxyD is primed for interaction with the large PCP-bound tyrosine substrate and requires a more open orientation for its catalytic function [237]. Interestingly, the substrate specificity conferred by the adjacent NRPS, which is thought to be involved in controlling the amino acid flux into secondary metabolism [237], may be useful for OxyD's biotechnological exploitation. OxyD and other similar amino acyl-PCP-oxidizing P450s do not display significant specificities for the free substrate, but instead require the presence of a PCP to deliver the bound substrate. Presumably, engineering these relatively nonspecific P450s to oxidize molecules presented on PCP scaffolds may provide a novel route to produce desired metabolites and hydroxylated cores of novel antibiotics [251].

The final example of the diverse biosynthetic P450s discussed here is the *R. erythropolis JCM 6824* P450 RauA (CYP1050A1) involved in the production of aurachin RE (Fig. 6.20a), a relatively new quinolone antibiotic [337]. Aurachin RE has broad antibiotic activity against a range of Gram-positive bacteria [338] and was recently revealed to be an inhibitor the *M. tuberculosis* 1,4-dihydroxy-2-naphthoate prenyltransferase (MenA), an essential menaquinone biosynthetic enzyme [339–342]. Menaquinone is an important/essential component of electron transport and respiration in a number of bacteria [341]. Aurachin RE is a rare alkaloid antibiotic with the structure incorporating a quinolone ring and farnesyl chain and bears similarity to menaquinone structures (Fig. 6.20). RauA catalyzes the N-hydroxylation of the quinolone ring of a biosynthetic intermediate to produce the active alkaloid antibiotic aurachin RE (Fig. 6.20a) [337]. RauA is a unique P450 with a single ortholog (CYP1050B1) identified in *Streptomyces*

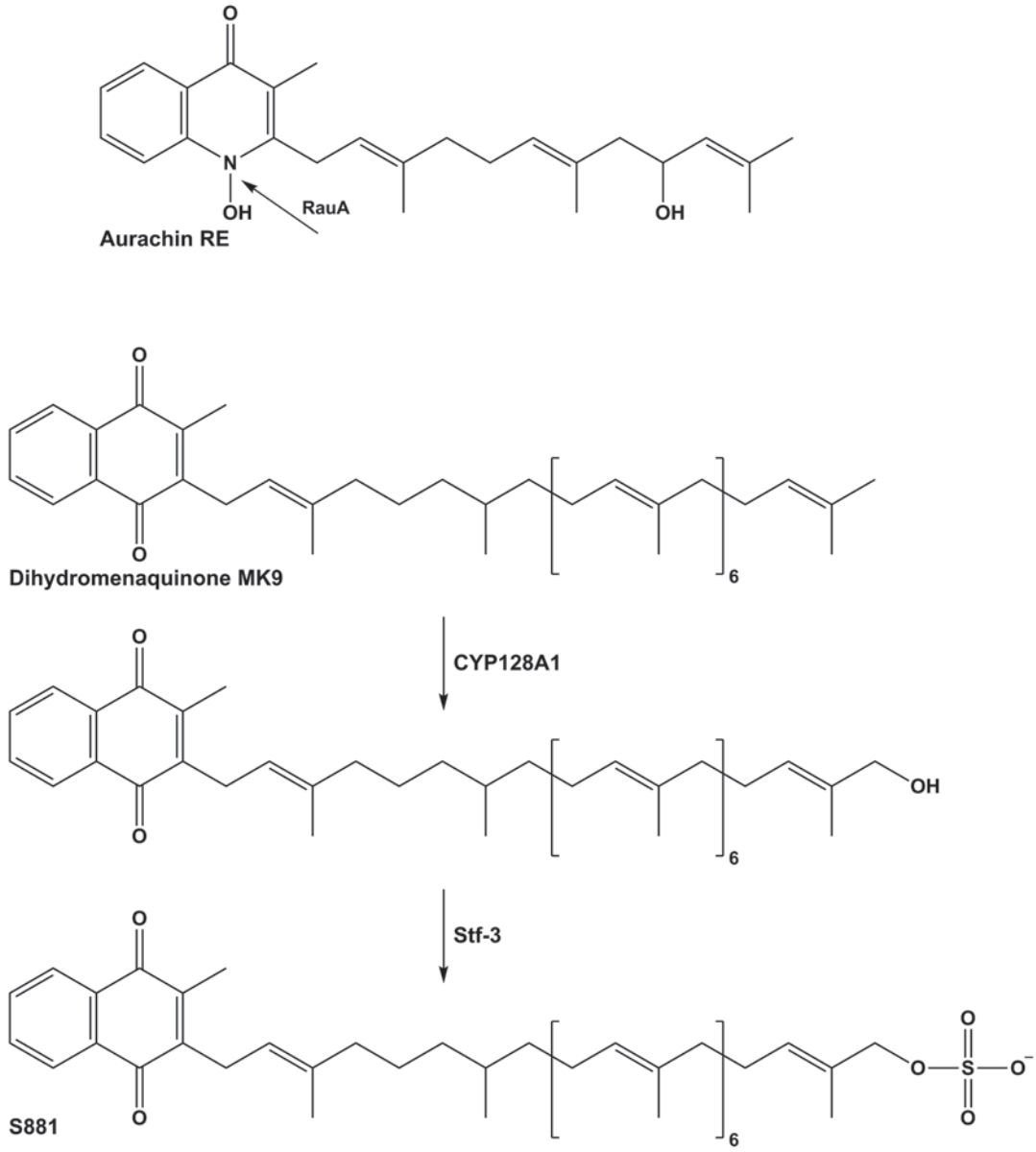

Fig. 6.20 Aurachin RE a tuberculosis (TB) drug, and oxidative modification of menaquinone by *M. tuberculosis* CYP128A1. **a** Aurachin RE an alkaloid antibiotic with anti-TB activity through its inhibition of menaquinone biosynthesis (enzyme MenA) [339]. *R. erythropolis* RauA (CYP1050A1) catalyzes the N-hydroxylation of an aurachin intermediate to produce the active aurachin RE compound [337]. **b** Dihydromenaquinone MK9, the major quinol electron carrier in Mtb respiration, is likely to be ω-hydroxylated by CYP128A1 (product of gene *Rv2268c*) prior to its sulfation by the sulfotransferase (stf-3, product of *Rv2269c*) encoded by the adjacent gene. The stf-3 reaction occurs at the hydroxyl group introduced by the P450, to produce the sulfated form of dihydromenaquinone MK9 (S881) [353]. S881 was shown to be associated with the outer cell membrane of Mtb, and to have a role as a negative modulator of virulence in a mouse model of infection [354]

sulphureus L180 with 46% identity [19]. BLAST searches also identify a CYP1050B1 ortholog from *Streptomyces roseochromogenes* subsp. *oscitans* DS 12.976 with 45% identity to RauA. Interestingly, the myxobacteria *Stigmatella* spp. are the only other species aside from *Rhodococcus* known to produce aurachin alkaloid antibiotics [343, 344]. However, the genome sequence of *Stigmatella aurantiaca* Sg-a15 does not contain a RauA-like P450 within the aurachin biosynthetic genes and has been shown to utilize a Rieske (2Fe–2S) oxygenase to perform the equivalent N-hydroxylation reaction [345]. RauA is thus exclusive and essential for production of aurachin RE in *R. erythropolis* [337]. The structure of RauA has been determined with its substrate, an Aurachin RE intermediate (3-[(2E,6E,9R)-9-hydroxy-3,7,11-trimethyldodeca-2,6,10-trien-1-yl]-2-methylquinolin-4(1H)-one) (PDB 3WEC) [346]. The active site cavity of RauA is hydrophobic with interactions between the substrate and the hydrophobic amino acid side chains, e.g., Leu77, Phe68, Phe74, Phe88, Leu89, Ile188, Phe190, and Ile399. The substrate farnesyl chain moiety extends upwards in the active site cavity and orients into a U-shaped conformation. Hydrophobic interaction between the middle of the farnesyl chain and the FG and BC loop close the active site cavity. The quinolone ring of the aurachin RE intermediate lies parallel to the heme place with the nitrogen situated immediately above the heme iron (4.3 Å), consistent with the RauA N-hydroxylation activity (Fig. 6.20a) [346]. The distal water ligand is retained, and correlates with spectroscopic data that do not show full conversion to the high-spin spectral species upon substrate binding. This active site water (WAT601) serves as a bridging molecule between the heme iron and the quinolone nitrogen but it is unclear whether it remains during oxygen binding and catalysis [346]. The structural characterization of a new biosynthetic P450 RauA and its N-hydroxylating role in the production of the active Aurachin RE drug may lead to the synthesis of novel alkaloid antibiotics. This is of particular interest with the antibacterial and anti-tuberculosis (TB) activities of this new class of aurachin compound.

In recent years, the study of P450s from diverse *Streptomyces* and other actinomycetes has unveiled several biosynthetic P450s with roles in the synthesis of compounds of interest in health, agriculture, and biotechnology. The characterizations of these P450s have not only given an understanding of the complex mechanisms involved in the biosynthesis of natural products but also provided strategies by which researchers might manipulate these enzymes and their associated pathways to produce new therapeutics and other desired compounds. Furthermore, as more genome sequencing data become available, there will undoubtedly be new P450-dependent pathways revealed, including novel P450s enzymes that perform unexpected chemistry. The volume and catalytic diversity of P450s in the actinomycetes should thus provide numerous further P450s for biomedical and biotechnological applications.

6.2.3.3 Mycobacterial P450s

In contrast to the metabolic gene organization observed in the *Streptomyces* and other actinomycetes, the mycobacterial *CYP* genes are often dispersed widely across their genomes and, with only a few exceptions, their genomic localizations give little or no clue towards their catalytic functions. Many of the mycobacterial P450s are located close (or adjacent) to 'conserved hypothetical protein' genes that are generally specific to the mycobacteria, but have no established function to date [59]. Those mycobacterial P450s that have been characterized are predominantly from the pathogenic bacterium *Mycobacterium tuberculosis* (Mtb) and have been shown to have a diverse range of substrates and functions [144, 347], as summarized in Table 6.4. Three Mtb P450s (CYP128, CYP125, and CYP121) were experimentally demonstrated to play essential roles in Mtb by different methods (including gene deletion studies). The *CYP125* gene is not essential for growth in vitro, but is required for survival of Mtb in the host, pointing to the importance of investigating a wide variety of conditions in order to discover genes that are important or essential during the adaptive phases of Mtb infection, persistence, and virulence [348–350].

Table 6.4 Properties of the Mtb P450s. Key facts are included that highlight experimental data from a number of genetic (transcriptomics, transposon mutagenesis, and microarray studies) and biochemical studies. Mtb P450s that have been structurally characterized are highlighted in light gray

P450/gene	Microarray/genetic analysis	Key facts
CYP121A1 (Rv2276)	Essential gene [350]. Possible virulence role with ΔAraC/XylS gene regulator mutant (ΔRv1931c). Induced in isoniazid and thiolactomycin-treated Mtb [807]. Clinical CYP121 deletion strains (RD182, 182a) isolated [391], but CYP121 consistently expressed among 10 Mtb clinical isolates [392]	Nanomolar azole drug affinity [350]. Operon with adjacent cylclopeptide synthase (Rv2275). Makes mycocyclosin from C–C coupling of cYY [65]. Structurally characterized (e.g., [65, 350])
CYP123A1 (Rv0766c)	Nonessential gene for Mtb H37Rv growth in vitro [374]. Upregulated at high temperatures [808] and mRNA levels higher than ΔPhoP virulence regulator [809]. Expressed in dormancy model [810] and protein detected in membrane fraction [811]	Possible operon with sterol demethylase CYP51B1 (Rv0764c) and adjacent 3Fe–4S ferredoxin (Rv0763c) [21]. Orphan P450 in terms of unknown enzyme function
CYP124A1 (Rv2266)	Nonessential gene for Mtb H37Rv growth in vitro [374]. Low expression in 10 Mtb clinical isolates [392]. Expressed in dormancy model [810]. Expression repressed in infected mouse [812] and upregulated in lupelone-treated Mtb [352]. Detected in Mtb whole cell lysates [813]	Possible operon with menaquinone MK9 sulfotransferase (Stf3, Rv2267c) [353, 354] and CYP128A1. Omega hydroxylates methyl-branched fatty acids and cholesterol/cholest-4-en-3-one. Structurally characterized [395]
CYP125A1 (Rv3545c)	Essential for infection in mice [376] and induced in macrophages [377]. In KstR reglon [406] and igr operon, essential gene for growth and virulence in macrophages and mice [380]. Expressed in dormancy model [810] and upregulated during infection of dendritic cells [378]	Part of igr operon with fadE28, fadE29, IgrD-E, and ltp2 (Rv3544c-3540c) [356, 380]. Cholesterol/cholest-4-en-3-one 26-oxidase. Structurally characterized (e.g., [360, 363])
CYP126A1 (Rv0778)	Nonessential gene for Mtb H37Rv growth in vitro [348]	Possible operon with essential purine biosynthesis genes, e.g., PurB adenylosuccinate lyase (Rv0777) and PurC phosphoribosylaminoimidazole-succinocarboxamide synthase (Rv0780). Orphan P450
CYP128A1 (Rv2268c)	Essential gene for Mtb growth [348]. Upregulated after starvation [351] and following lupelone treatment of Mtb [352]	Operon with adjacent menaquinone MK9 sulfotransferase (Stf3,Rv2267c) and possibly CYP124A1. Likely MK9 hydroxylase prior to Stf3 sulfation [353, 354]
CYP130A1 (Rv1256c)	Nonessential gene [348]. Absent from M. bovis and M. bovis BCG (RD13 (10)) [421] ([422]). Expressed in Mtb dormancy model [810]	Structures determined for ligand-free monomer and econazole-bound dimer [409]. Orphan P450
CYP132A1 (Rv1394c)	Nonessential gene [348]. Transcription controlled by adjacent AraC (Rv1395c) transcriptional regulator with virulence-related role [814]. Induced following diamide oxidative stress [815] and upregulated during infection of dendritic cells [378]. Expressed in dormancy model [810]	Similarities in protein sequence to fatty acid metabolizing P450s and CYP4 family. Orphan P450
CYP135A1 (Rv0327c)	Nonessential gene [431]. Induced following diamide stress [815]	Orphan P450
CYP135B1 (Rv0568)	Nonessential gene [348]. Detected in Mtb cytosol [811]. Low expression in 10 Mtb clinical isolates [392]. Expressed in Mtb dormancy model [810]	Orphan P450

Table 6.4 (continued)

P450/gene	Microarray/genetic analysis	Key facts
CYP136A1 (Rv3059)	Nonessential gene [348]. Expressed in Mtb dormancy model [810]	Weakly related to sterol demethylase CYP51 family. Close to TetR transcriptional regulators and acyl-coA dehydrogenase fadE22 (Rv3061c). Orphan P450
CYP137A1 (Rv3685c)	Nonessential gene [348]. Detected in Mtb membrane fraction [811]. Downregulated following lupelone treatment of Mtb [352]	Orphan P450
CYP138A1 (Rv0136)	Nonessential gene [348]. Upregulated at high temperatures [808], in presence of lung surfactant [816], during iron limitation [817] and following lupelone treatment of Mtb [352]. Low expression observed in 10 Mtb clinical isolates [392]	Adjacent to putative transcriptional regulator (Rv0135c). Orphan P450
CYP139A1 (Rv1666c)	Nonessential gene [348]	Adjacent to polyketide synthase genes (pks 10,7,8,17,9,11) (Rv1660–1665) and to macrolide transport genes (Rv1667c-1668c). Orphan P450
CYP140A1 (Rv1880c)	Nonessential gene [348]. Expressed in Mtb dormancy model [810], Upregulated following lupelone treatment of Mtb [352]	Closest Mtb relative to sole M. leprae P450 (CYP164A1). Orphan P450
CYP141A1 (Rv3121)	Absent from M. bovis and M. bovis BCG strains (RD12 (5)) [421, 422]. Upregulated in presence of lung surfactant [816]	Surrounding genes involved in molybdenum cofactor biosynthesis. Orphan P450
CYP142 (Rv3518c)	Nonessential gene [348]. Expressed in Mtb dormancy model [810] Located in KstR reglon [406]. Detected in cell wall fraction [811]. Pseudogene in M. bovis and M. bovis BCG due to a 2-bp deletion	Cholesterol/cholest-4-en-3-one 26-oxidase [362, 364]. Structurally characterized P450 enzyme [362]
CYP143 (Rv1785c)	Nonessential gene [348]. Low expression in 10 Mtb clinical isolates [392]. Deleted in M. smegmatis (region 5)	Adjacent to 3Fe–4S ferredoxin (Rv1786) [818]
CYP144A1 (Rv1777)	Nonessential gene [348]. Expressed in Mtb dormancy model [810]. Upregulated during infection of dendritic cells [378]	Tight azole drug binding [819]
CYP51B1 (Rv0764c)	Nonessential gene [348]. Possible role in host sterol/steroid metabolism. Expressed in dormancy model [810]	Tight azole drug binding. Adjacent to 3Fe–4S ferredoxin (Rv0763c). Sterol 14α-demethylase activity [430]. Possible role in host sterol/steroid metabolism

mRNA messenger RNA

These three *CYP* genes are also among the few Mtb P450s to give a clue to their roles from their genetic context.

CYP128A1 was the only P450 identified as essential for optimal growth of the pathogenic Mtb H37Rv strain under normal laboratory conditions in a genome-wide transposon hybridization study (TraSH) [348]. Microarray analysis also identified the expression of the *CYP128A1* (*Rv2268c*) gene as upregulated after nutrient starvation [351], as well as following exposure to the potential anti-TB drug lupulone [352]. *CYP128A1* is located chromosomally adjacent to a sulfotransferase (stf-3, *Rv2269c*) that has a unique role in the modification of dihydromenaquinone MK9, the major quinol electron carrier in Mtb respiration, with sulfation at the ω-position of its polyisoprenoid chain [353]. CYP128A1 was predicted to catalyze an ω-hydroxylation of dihydromenaquinone MK9 that subsequently allows the stf-3-catalyzed menaquinone sulfation reaction to occur (Fig. 6.20b). The sulfated form

of the dihydromenaquinone MK9 (named S881) was shown to be associated with the outer cell membrane of Mtb, with a potential role as a negative modulator of virulence in a mouse model of infection [354]. Sulfated lipids in Mtb were shown to have important functions in virulence and also to mediate specific host–pathogen interactions during infection. Furthermore, S881 may potentially be involved in the regulation of the Mtb internal menaquinone pool, and thus have an important role in regulating Mtb respiration [353–355]. Attempts to purify and characterize the CYP128 P450 have proven unsuccessful to date due to its insolubility. However, the hydrophobic nature of the putative dihydromenaquinone MK9 substrate suggests that it may be membrane associated [144]. The CYP128 family is uniquely restricted to the pathogenic Mtb family that includes other Mtb strains and the closely related *M. bovis*.

The second essential Mtb P450 is CYP125A1, a cholesterol oxidase with interesting catalytic properties. The catabolism of cholesterol was shown to be important for survival of pathogenic mycobacteria in the host [356–358]. *CYP125A1* along with *CYP142A1* (which can compensate for defects in *CYP125A1*) are located in a large regulon with multiple other genes that encode different enzyme components of the cholesterol degradation pathway. The identification of the role of this operon came following the functional description of a related gene cluster for cholesterol catabolism in the soil bacterium *Rhodococcus jostii* RHA1 [359]. The Mtb CYP125A1 and CYP142A1 enzymes both catalyze C26 ω-hydroxylation(s) of the side chain of cholesterol, and of its ketone derivative cholest-4-en-3-one, in a primary step towards the breakdown of the cholesterol side chain and the catabolism of cholesterol [360–364] (Fig. 6.21a). Intriguingly, CYP125A1 was also discovered to simultaneously produce five additional products, resulting from deformylation of the aliphatic cholesterol side-chain aldehyde intermediate (Fig. 6.21b) [365]. One of the products of this unusual rearrangement and C–C bond cleavage reaction is an atypical formyl ester (27-*nor*-25-oxyformyl-cholest-4-en-3-one/cholesterol) (Fig. 6.21b (M2)), highlighting an uncommon diversity of CYP125A1 products, produced by consecutive catalytic turnovers with a single substrate [186, 365]. The functional relevance of these molecules is currently unknown; however, cholesteryl esters are known to accumulate in Mtb-infected human macrophages [366]. Successive cholesterol oxidations resulting in the conventional hydroxy-, aldehyde-, and acid- cholesterol/one derivatives has also been observed for CYP125 enzymes from other nonpathogenic mycobacteria and *Rhodococcus sp.* [367, 368]. Following identification of the cholesterol regulon in *Rhodococcus jostii* RHA1, similar gene clusters have been identified in a growing number of *Rhodococcus, Gordonia,* and *Tsukamurella* spp. [359, 369–371]. However, cholesterol is not synthesized de novo in these organisms and is generally recruited from the cholesterol-rich host immune system in the response following TB infection [372, 373], or else is taken up from the environment (e.g., soil) by nonpathogenic organisms, where cholesterol catabolism can detoxify environmental steroids or provide energy to aid cellular growth [370]. Cholesterol was also shown to be one of the major bacterial carbon sources during infection by pathogenic Mtb and there is a growing body of evidence in the literature relating to the importance of cholesterol metabolism in Mtb virulence and pathogenesis throughout the course of clinical infection and disease [356–358, 374, 375]. The *CYP125A1* gene was shown to be the only *CYP* gene that is both essential in vivo for Mtb infection in mice and induced in Mtb-infected human macrophages [349, 376, 377]. *CYP125A1* is also upregulated in Mtb-infected human dendritic cells: the antigen-presenting cells that play a key role in host cell immunity as well as Mtb pathogenicity [378, 379]. Furthermore, *CYP125A1* is a member of the intracellular growth (igr) region that is essential for growth and virulence in macrophages and in mice, and necessary for degradation of the cholesterol 2′-propanoate side chain. The igr consists of *CYP125A1* (*Rv3545c, igrA*), the acyl coA dehydrogenases *fadE28* and *fadE29* (*Rv3544c* and *Rv3543c, igrBC*), a conserved hypothetical protein (*Rv3542c, igrD*), a likely enoyl coA hydratase (*Rv3541c, igrE*) and a lipid carrier protein *ltp2* (*Rv3540c, igrF*) [356, 380, 381]. It

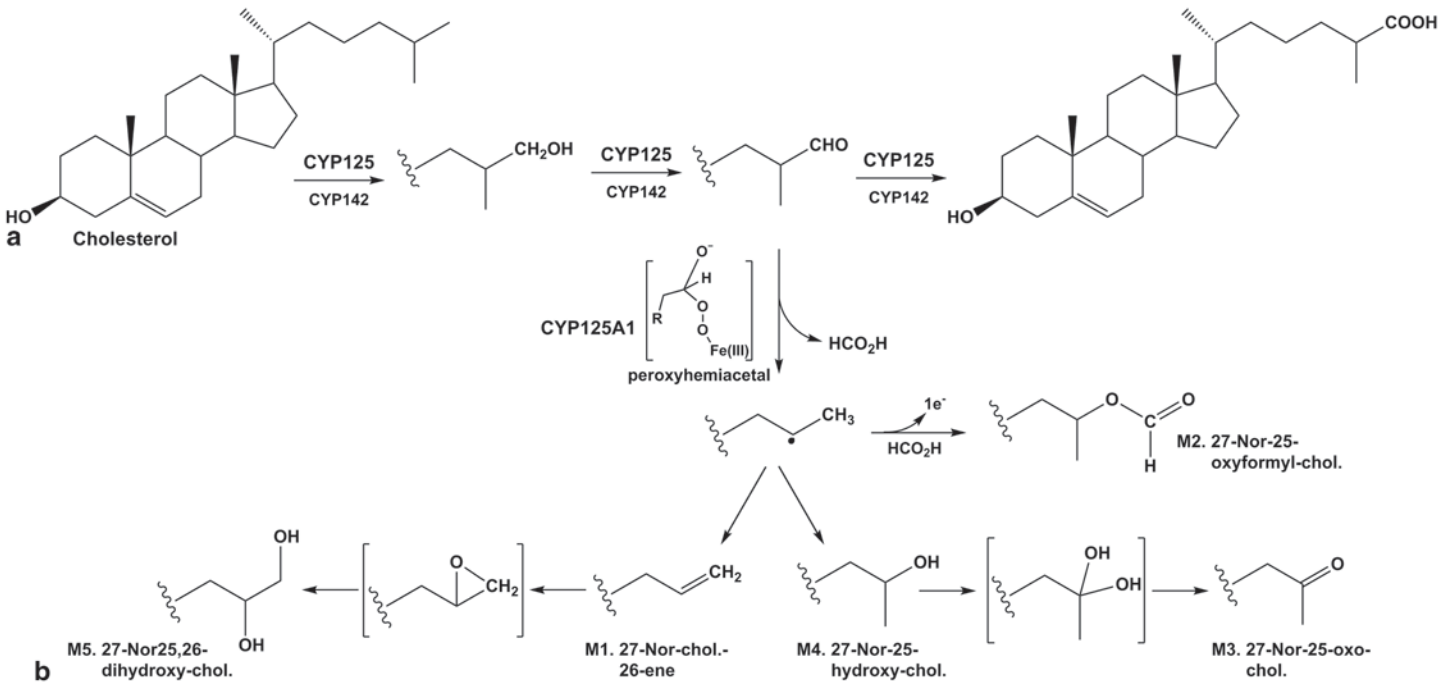

Fig. 6.21 The oxidation of cholesterol and cholesten-4-en-3-one. **a** The CYP125A1/CYP142A1 (and CYP124A1)-dependent conversion of cholesterol and cholesten-4-en-3-one through C26-oxidation reactions to the acid via the hydroxyl and aldehyde forms [360–364]. **b** CYP125A1-catalyzed deformylation of the side chain of cholesterol and cholesten-4-en-3-one (chol.) via a peroxyhemiacetal adduct, predicted to be derived from the reaction of the heme iron ferric–peroxo anion ($Fe(III)O_2^-$) species with the aldehyde intermediate, leading to C–C bond cleavage. The observed products and proposed reaction mechanisms include radical fragmentation of the peroxyhemiacetal adduct, leading to formation of an alkene (M1, 27-Nor-chol.-ene) or a one-carbon-deficient alcohol (M4, 27-Nor-25-hydroxy-chol.). A diol (M5, 27-Nor-25,26-dihydroxy-chol) is formed via the acid-catalyzed ring opening of an epoxide intermediate that is generated by the oxidation of M1 (utilizing CYP125A1 compound I). Oxidation of M4 (also utilizing compound I) leads to a keto-compound (M3, 27-Nor-25-oxo-chol.) via dehydration of a gem-diol intermediate. The formation of the unusual C25 oxyformyl product (M2, 27-Nor-25-oxyformyl-chol.) occurs via a single-electron oxidation of the radical to form a cation that is trapped by formate (or water). An alternative mechanism to form M2 has also been proposed, involving a Baeyer–Villiger oxidation with the ferric–peroxo anion species (not shown) [186, 365]

is now generally considered that CYP125A1 is the major cholesterol oxidase P450 and has an important adaptive role in the utilization of host cholesterol for catabolism, the detoxification of cellular cholest-4-en-3-one, and potentially also for the cholesterol-derived synthesis of the important cell wall lipid phthiocerol dimycoserate (PDIM) [363, 364, 382, 383]. Recently, one of the CYP125/CYP142 products 3-oxo-4-cholestenoic acid, produced by three successive oxidations of cholest-4-en-3-one, was shown to be an inducer of one of the two TetR-type transcriptional repressors (KstR) that regulates part of the cholesterol catabolic gene cluster in *M. smegmatis* [384, 385]. Although it cannot be ruled out that other oxidized products from the cholesterol pathway may also act as inducers, these studies highlight a further important role of the cholesterol oxidase P450s [384]. The CYP125 family extends from pathogenic and nonpathogenic *Mycobacterium* spp. to other more diverse actinomycetes, such as certain *Streptomyces, Rhodococcus,* and *Salinospora* spp., and a CYP125 ortholog is also identified in the myxobacterium *Sorangium cellulosum Soce56* (CYP125E1). The second cholesterol oxidase in Mtb (CYP142A1) has similar genetic diversity to CYP125A1. However, in some clinical Mtb and *M. bovis* strains, as well as in the vaccine strain *M. bovis* BCG, *CYP142A1* exists as a pseudogene, rendering the loss of *CYP125* lethal to intracellular bacteria without the compensatory CYP142A1 enzyme present. These data highlight the important role of CYP142 in certain Mtb strains, possibly as an evolutionary adaptation to ensure cholesterol catabolism can occur during pathogenesis. In this scenario, CYP142A1 may act as a secondary catalyst for energy generation from cholesterol/cholestenone, and cooperate with CYP125A1 rather than playing a direct role in Mtb virulence [361–364]. Despite having a similar substrate-oxidizing role to CYP125A1, the protein sequences of the CYP142 cholesterol oxidases display low levels of identity (~28%) with CYP125A1. The structures of these genes from Mtb and the nonpathogenic *M. smegmatis* are discussed below in more detail.

CYP121A1 (*Rv2276*) is another example of the diverse functions of the Mtb P450s and is the third essential gene in Mtb H37Rv. *CYP121A1* is located adjacent on the genome to a cyclic dipeptide (CDP) synthase (*Rv2275*) that produces the CYP121 substrate cyclo-*L*-tyrosine-*L*-tyrosine (cYY) using two molecules of the amino acyl tRNA derivatives of *L*-tyrosine. CYP121A1 then catalyzes C–C bond formation by oxidative coupling of the cYY aryl side chains to make a metabolite named mycocyclosin (Fig. 6.22a) [65, 386]. The physiological role of mycocyclosin is yet to be determined, but members of this diketopiperazine class of compounds have been found to play important roles in, e.g., immunosuppression and blockage of cation channels, and possibly as toxins [387–390]. *CYP121A1* was shown to be essential for viability in Mtb through genetic studies involving construction of a chromosomal *CYP121A1* gene insertional knockout mutant through a two-step homologous recombination process. It proved possible to delete *CYP121A1* only when a second version of the gene was integrated elsewhere on the chromosome, confirming its essentiality for Mtb viability [350]. However, Mtb clinical isolates have been described that have full or partial deletions of *CYP121A1* and its neighboring genes. The physiological effects on these deletion strains are unknown, but it has been speculated that loss of *CYP121* is likely deleterious to Mtb. Potentially, these types of deletions may confer a short-term evolutionary advantage, such as curtailing latency, evading the host immune system or providing antibiotic resistance, which may be advantageous to the pathogen at certain stages in infection [391]. Other studies have identified *CYP121A1* as the only P450 among the 16% of genes in Mtb that are consistently expressed across a panel of clinical strains, thus highlighting the importance of *CYP121A1* and these other genes in the viability of the Mtb isolates [392]. Like *CYP128,* the *CYP121* gene family is also exclusively found in the pathogenic Mtb spp. Interestingly, a similar genomic orientation of a CDP synthase (*YvmC*) and a P450 (*CYP134A1*) was identified in *B. subtilis* [393]. These enzymes were shown to be involved in successive steps producing the CDP cyclo-*L*-leucine-*L*-leucine (cLL), followed by a three-step CYP134A1-mediated oxidation of the

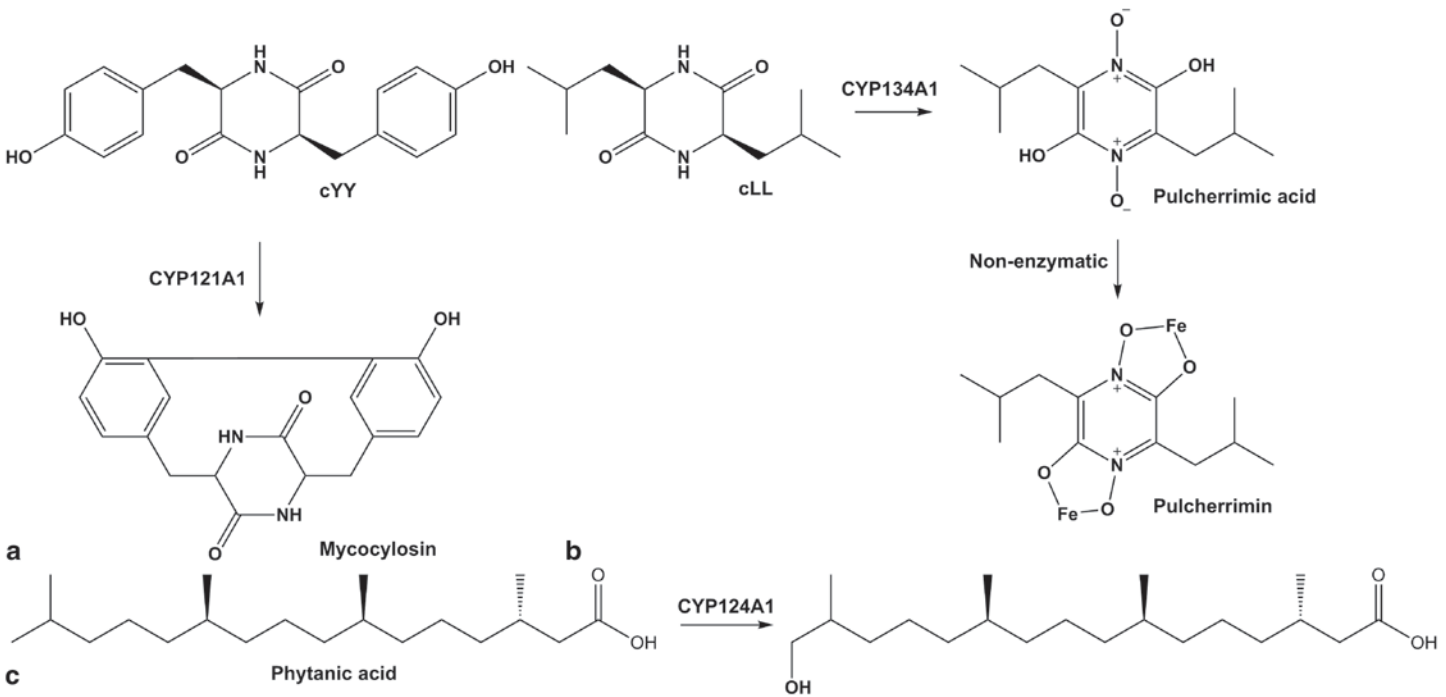

Fig. 6.22 Reactions catalyzed by the Mtb P450s CYP121A1, CYP134A1, and CYP124A1. **a** CYP121A1 catalyzes the C–C bond coupling of the substrate cyclo-*L*-tyrosine-*L*-tyrosine (cYY) to from a product named mycocyclosin. cYY is produced by the adjacent cyclodipeptide synthase (product of gene *Rv2275*) using two molecules of the amino acyl tRNA derivatives of *L*-tyrosine [65]. **b** The three-step oxidative transformation of the diketopiperazine cyclo-*L*-leucine-*L*-leucine (cLL) into pulcherriminic acid catalyzed by the *B. subtilis* CYP134A1 involved in the production of the pigment pulcherrimin [393]. **c** The *omega* hydroxylation of phytanic acid by CYP124A1. CYP124A1 catalyzes the hydroxylation of a variety of methyl-branched chain fatty acids as well as the oxidation of the hydrocarbon chain of cholesterol and cholest-4-en-3-one [395]

CDP to produce pulcherriminic acid, a precursor of the extracellular iron-chelating pigment pulcherrimin that is thought to play a role in ultraviolet (UV) protection [393, 394] (Fig. 6.22b). However, CYP134A1 is not highly related to CYP121A1 and performs a different noncoupling reaction, reflecting the distinct physiological functions of these two pathways.

CYP124A1 (*Rv2266*) is apparently a nonessential P450 gene that directly precedes the *CYP128A1* operon that contains the menaquinone MK9 ω-sulfotransferase (Sft3, *Rv2267c*) with a likely role in bacterial virulence [353, 354]. CYP124A1 preferentially catalyzes the ω-hydroxylation of methyl-branched lipids such as phytanic acid (Fig. 6.22c) and it is postulated that it may have an as yet uncharacterized role in the oxidation of lipids similar to menaquinone MK9, and possible functions in the generation of sulfolipid derivatives [395]. Furthermore, similar to what was observed for *CYP128A1,* the expression of *CYP124A1* is also upregulated following exposure to the potential anti-TB drug lupulone [352]. The *CYP124* gene family extends across the actinomycetes, with many members in the *Streptomyces*. Interestingly, the CYP124A1 protein does have considerable similarity to CYP125A1 (40.1% identity), indicating evolutionary relationships, and can also catalyze C26 *omega*-hydroxylation of cholesterol and cholest-4-en-3-one (see below).

CYP139A1 is a further P450 enzyme unique to the pathogenic Mtb bacteria, although it appears to be nonessential for Mtb viability under standard laboratory conditions [348, 374]. However, the genomic localization of *CYP139A1* provides clues to its physiological role. *CYP139A1* is located at the end of a large gene cluster of polyketide synthase (PKS) genes (PKS 10, 7, 8, 17, 9, 11), with PKS 7 and 8 being identified as essential genes in Mtb [348, 374]. *CYP139A1* is also located immediately upstream of two putative macrolide transporters, and a number of arginine biosynthetic genes also precede the PKS gene cluster [396]. It is thus tempting to speculate that CYP139A1 may be involved in a biosynthetic operon involved in the production of an Mtb macrolide compound. Thus, there is a potential role for CYP139A1 in the oxidative tailoring of a macrolide, as is observed with many *Streptomyces* P450s. CYP139A1 has yet to be characterized, but it will be interesting to establish if this P450 plays a novel role in oxidation of a Mtb secondary metabolite.

The Mtb-related pathogen *M. ulcerans* Agy99 is the causative agent of Buruli ulcer, a debilitating, necrotizing ulcerative disease common in equatorial Africa, but also identified in Asia, Australia, and South America [397–400]. CYP140A7 (encoded by the *mup053* gene) is one of the 21 *M. ulcerans* P450s, and further highlights the functional diversity of mycobacterial P450s. CYP140A7 is implicated in the synthesis and structural diversification of mycolactone A/B (Table 6.1; Fig. 6.23). Mycolactone A/B is the major member of a diverse group of macrolide toxins responsible for the clinical characteristics and virulence of *M. ulcerans* [398, 399, 401, 402]. The mycolactones differ mainly in the heterogeneity of the fatty acid side chains around the lactone core and were shown to have distinctive cytotoxic, apoptotic, and immunosuppressive properties [397, 403]. Their mode of action is through the downregulation of specific proteins implicated in important cellular processes such as immune response and cell adhesion, and through the disruption of protein translocation into the endoplasmic reticulum [400]. CYP140A7 is exclusive to pathogenic mycobacteria (including Mtb), with closely related orthologs in *M. xenopi* and *M. avium* spp., and in other organisms that cause disease in, e.g., humans, fish, and frogs [66, 400, 404]. *CYP140A7* is present on a megaplasmid that contains three extremely large type I PKSs (MLSA1, MLSA2, and MLSB) that each contain several modules and the enzymatic activities required to produce the C1–C20 lactone core (MLSA) and the C1′–C16′ side chain that are subsequently esterified to form Mycolactone C, likely catalyzed by a ketosynthase (*mup045*). CYP140A7 performs the final synthetic step, catalyzing the C12′ hydroxylation to produce mycolactone A/B [398, 399, 404]. Mycolactone A/B exists in a dynamic equilibrium between two geometric *Z* and *E* isomers at the C4′–C5′ position on the polyketide chain, with the *Z* isomer

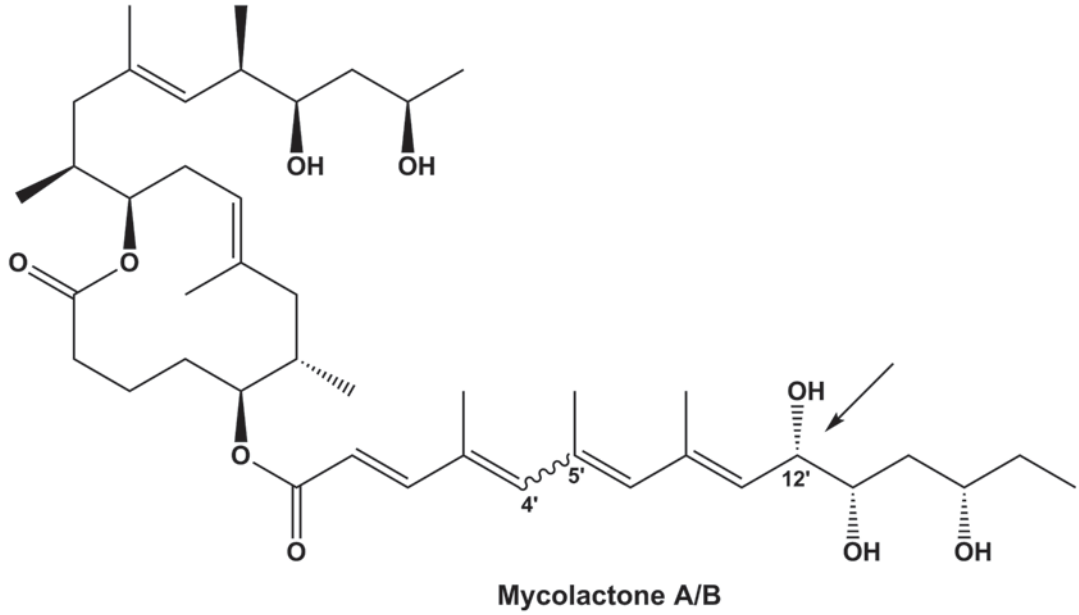

Fig. 6.23 The structure of mycolactone A/B. Mycolactone is an immunosuppressant toxin produced by selected pathogenic mycobacterial strains and is responsible for the formation of Buruli ulcers. A crucial role exists for a P450 in synthesis of the toxin. Mycolactone A/B is formed through the *M. ulcerans* CYP140A7-mediated C12′-hydroxylation shown highlighted with an *arrow*. Mycolactone A/B exists in a dynamic equilibrium between two geometric *Z* and *E* isomers at the C4′–C5′ position on the polyketide chain, with the *Z* isomer (mycolactone A) predominant over the corresponding *E* isomer (mycolactone B) [399, 404]

(mycolactone A) predominant over the corresponding *E* isomer (mycolactone B) [399, 404]. Further, structural characterization of CYP140A7 will be of interest to reveal details of the substrate and product interactions with the P450, and to give insights into the mechanism of diversification of the different mycolactone forms.

A high proportion of the mycobacterial P450s are orphan enzymes with no known function, and in most such cases genomic localization does not give any clear indication as to what their physiological or infection-related roles might be. In the case of the pathogen Mtb, a number of in vivo and in vitro transcriptomic and microarray experiments have revealed genes that display changes in their expression levels upon exposure to conditions associated with bacterial growth, dormancy, or infection. Similar studies have been done to probe effects of various antibiotics and other drugs known to influence Mtb growth or viability (e.g., [351, 378, 405, 406]). Whilst these studies may not directly identify physiological roles for the P450s and other Mtb genes, they can identify conditions that may be relevant to the action of a particular enzyme (e.g., whether it is active during a certain phase of infection) and potentially highlight which enzymes have important roles in Mtb (Table 6.4). A number of the mycobacterial P450s have proven difficult to express and purify from a heterologous expression host (e.g., *E. coli*). This can be due to aspects such as the different nature of their natural cellular environments, their native association with the mycobacterial membrane and the high GC content of the mycobacterial genome compared to that of the host for recombinant expression (e.g., [144, 407]). This has hampered the structural and biochemical characterization of some of the mycobacterial P450s. However, careful control of recombinant cell growth and gene expression conditions along with efficient protein purification has allowed the purification and structural determination of a growing number of P450s from mycobacteria (e.g., [187, 408, 409]).

The cholesterol oxidase P450s are the largest group of structurally characterized mycobacterial enzymes. The structure of the major enzyme CYP125A1 was solved in the ligand-free (PDB 3IVY, 3IWO, and 2XN8) and the cholest-4-en-3-one substrate-bound (PDB 2X5W) forms [360, 363, 410]. In addition, there are also CYP125A1 structures with the inhibitors econazole and the nonazole inhibitor LP10-bound (PDB 3IW2 and 2XC3, respectively), as well as an androstenedione-bound structure (PDB 3IWI) [360, 410]. The ligand-free structures of CYP125A1 reveal a letterbox-like active site cavity between the central portion of the I-helix, the C-terminal loop, and the B′ and F helices along with the preceding loop region. The B and F helices define a narrowing cavity that funnels down from the protein surface to the heme and which is lined by hydrophobic residues. The catalytic site around the CYP125A1 heme iron and distal water consists of Leu117, Ala268, Val313, Phe316, and the methyl group of the conserved threonine Thr272 [360, 410]. The distal water is not present in all of the ligand-free structures, and this observation is consistent with the propensity of CYP125A1 to be purified in a predominantly high-spin form [360, 410]. The cholest-4-en-3-one-bound structure (Fig. 6.24a) reveals conformational changes in the H-helix and the N-terminal region of the I-helix that enclose the active site cavity and permit hydrophobic contacts between the I-helix and the substrate molecule [363]. The tetracyclic steroid ring system of cholest-4-en-3-one sits in the mouth of the active site access channel and makes van der Waals contacts with Val267 and Trp414 that prevent the steroid portion of the molecule further access toward the heme. The aliphatic side chain of cholest-4-en-3-one reaches towards the heme plane and is enclosed in the narrow active site, although in an orientation that would apparently disfavor C26 hydroxylation. It is thus postulated that a minor structural rearrangement would allow a catalytically productive substrate orientation [363]. This restriction and the narrowing of the active site access channel is also highlighted in the androstenedione- and econazole-bound CYP125A1 structures, which occupy a similar position to that seen for the steroid ring portion of cholest-4-en-3-one [360]. The binding of the inhibitor LP10 (α-[(4-methylcyclohexyl)carbonyl amino]-N-4-pyridinyl-1H-indole-3-propanamide), a type II inhibitor of *Trypanosoma cruzi* CYP51E, is also restricted by the funnelling of the active site access channel, and the majority of the molecule occupies the same region as that for the base of the cholest-4-en-3-one steroid ring. However, the pyridinyl ring of LP10 points into the heme pocket and hydrogen bonds with an active site water molecule adjacent to the axial water ligand to the heme iron [410]. The structure of the second Mtb cholesterol oxidase CYP142A1 was solved in the ligand-free form (PDB 2XKR) and displays the same type of letterbox-shaped access channel as described for CYP125A1, which is formed in CYP142A1 by the BC and FG loop, and the N-terminal region of the I-helix. However, there are significant differences in the FG helices and the loop region connecting the B and C helices, with the absence of an extended loop connecting the β1 and β2 strands compared to the CYP125 structure [362]. Interestingly, the structures of CYP125A1 and CYP142A1 display high degrees of similarity to that of CYP124A1, again providing insights that suggest a common evolutionary origin. The structure of CYP124A1 was determined in the ligand-free (PDB 2WM4) and phytanic acid substrate-bound (PDB 2WM5) forms [395]. Similar to CYP142A1, in CYP124A1 there is also the absence of the extended loop that is seen to connect the β1 and β2 regions in CYP125A1 [362]. Substrate binding to CYP124A1 induces a structural rearrangement of the FG helices and movement of the FG loop towards the phytanic acid ligand, closing over the access channel to the substrate cavity, as also observed in other P450 structures (e.g., [24, 112]). This is accompanied by reorientation of the EF loop, G loop, H-helix, and HI loop. The BC loop and the D and C helices also move toward the G-helix to accommodate closure of the active site. The phytanic acid molecule is bound in a conformation optimal for ω-hydroxylation, with hydrophobic and polar interactions observed between different secondary structural elements and the substrate methyl-branched lipid chain and carboxylic acid groups,

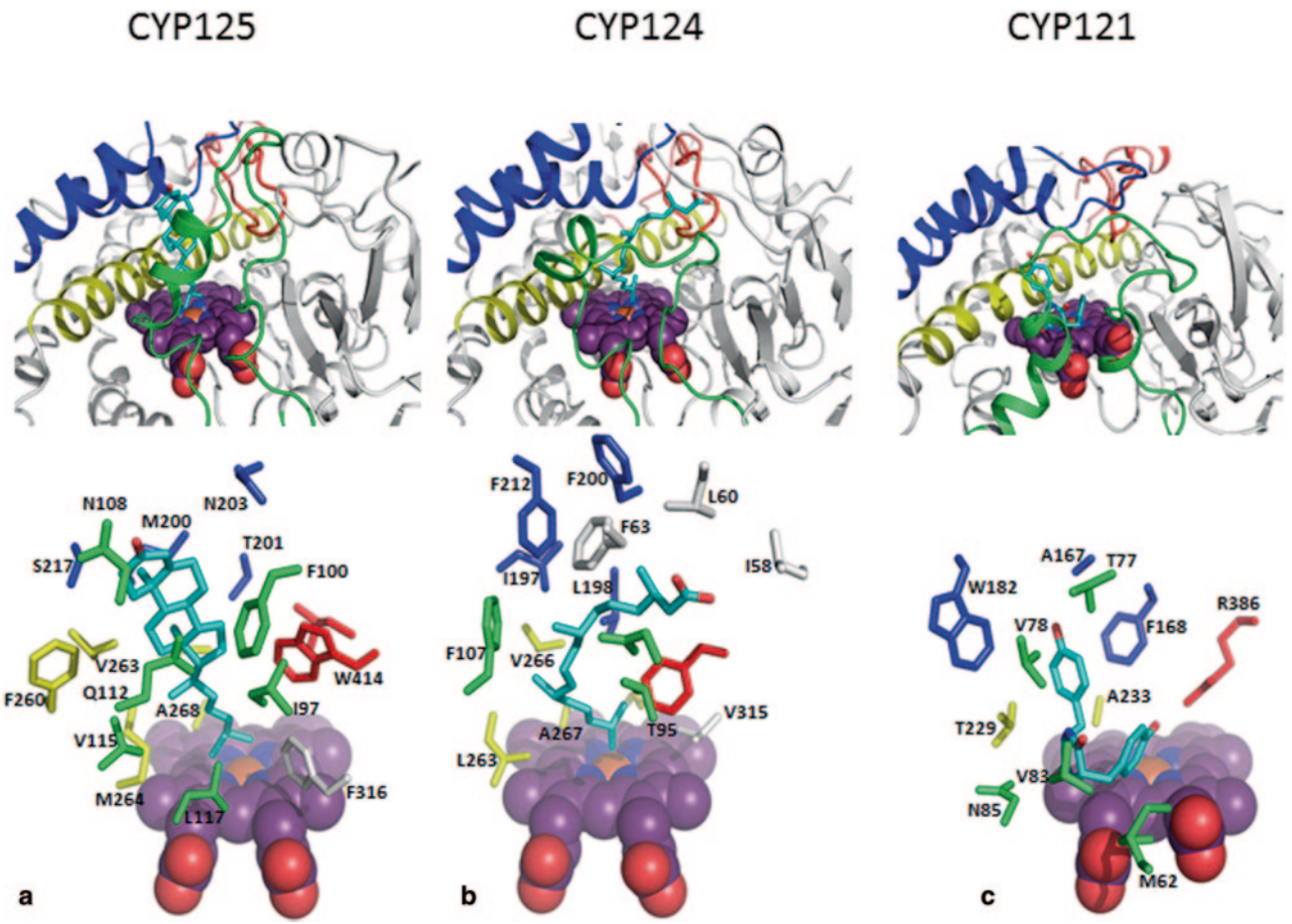

Fig. 6.24 Structures of substrate complexes of *M. tuberculosis* P450s. Expanded structural views of the active site regions (*upper* panel) and close-up views of the active site cavity organization (*lower* panel) of the CYP125A1 complex with cholest-4-en-3-one (**a**) (PBD 2X5W) [363]; the CYP124A1 complex with phytanic acid (**b**) (PDB 2WM4) [395]; and the CYP121A1 complex with cYY (**c**) (PDB 3G5F) [65]. Selected amino acids involved in substrate binding are shown in stick representation, color coded as in Fig. 6.1. The substrates are shown in atom colored sticks with cyan carbons

respectively (Fig. 6.24b). Additional solvent-filled pockets observed in the active site cavity of phytanic acid-bound CYP124A1 suggest that these regions are not occupied by phytanic acid, but may instead accommodate parts of as yet unknown physiological substrates of CYP124A1 [395]. CYP124A1 can also hydroxylate cholesterol and cholest-4-en-3-one at the C26 position. However, the fact that *CYP124A1* does not compensate for loss of *CYP125A1* function in Mtb (whereas *CYP142A1* does) probably means that these steroids are unlikely to be true physiological substrates of CYP124A1 [364, 395]. Instead, CYP124A1 is more likely to have a role in fatty acid metabolism given its preference for methyl-branched chain lipid substrates, and potentially might oxidize a menaquinone-type derivative or precursor, given its colocation with *CYP128A1* on the Mtb chromosome.

Regardless of their physiological functions, there is an intriguing structural relationship between these three enzymes that possess cholesterol oxidase activity. Indeed, the structure of CYP124A1 was solved by molecular replacement using the atomic coordinates of CYP125A1 as a search model [360], with an amino acid sequence identity of 40.1% between these P450s. Similarly, CYP142A1 was solved using the structure of CYP124A1 [362], with amino acid sequence identity of 36.1% between this pair of P450s. The lower identity between CYP125A1 and CYP142A1 (27.7%) means that the cholesterol oxidases CYP142A1 and CYP125A1 are both more similar to CYP124A1 than they are to each other. A structural comparison of the substrate-bound forms of CYP125A1 and CYP124A1 with the ligand-free CYP142A1 [362, 363, 395] revealed that CYP142A1 and CYP124A1 possess near-identical active site pockets immediately surrounding the heme, with conservation of the majority of the active site amino acid side chains in both identity and position. CYP142A1 and CYP125A1 do not bind productively to the methyl-branched lipids identified as ligands or substrates for CYP124A1 [362, 395]. CYP125A1 shows diversity in the structural composition of its equivalent distal heme pocket (compared to CYP142A1 and CYP124A1), despite it possessing similar cholesterol hydroxylation activities (Figs. 6.24a and b) [362]. In contrast, when structural comparisons are made between these P450s in regions slightly removed from the heme distal pocket, a much greater degree of structural similarity is revealed between the substrate access channels of CYP125A1 and CYP142A1. In this region, CYP124A1 exhibits a distinct type of channel, with its shape and positioning dissimilar to those of the other two P450s [362]. These comparative similarities and differences clearly point to evolutionary relationships, suggesting that CYP125A1/CYP142A1 and CYP124A1 may have evolved from a common progenitor to perform different roles and to enhance the ability of Mtb to adapt to availability of different lipid substrates, thus contributing to its success as a human pathogen. CYP125A1 and CYP142A1 have evolved to perform cholesterol/one oxidations, and difference in active site heme distal pockets between this pair of P450s potentially explains the additional capabilities of CYP125A1 to oxidize cholesterol and cholest-4-en-3-one for other purposes in Mtb infection, e.g., by synthesizing the recently identified additional CYP125A1 deformylation products from the aldehyde intermediate [365]. CYP124A1 likely has a different role in Mtb to the other two P450s, but the high level of structural conservation between CYP124A1 and CYP142A1 in their heme distal pockets likely explains the retention of cholesterol hydroxylase activity in CYP124A1. Thus, CYP124A1's main function may now relate to oxidation of branched chain or other lipids (rather than steroids), but its true physiological role is still to be established [364].

The CYP125 and CYP142 cholesterol oxidase orthologs from the fast-growing nonpathogenic *M. smegmatis* have also been characterized and are located on a similar cholesterol regulon that also contains the igr operon [411], as described for CYP125A1 in Mtb [356, 380]. The *M. smegmatis* CYP125A3 and CYP142A2 enzymes have similar biochemical properties to their Mtb counterparts and also catalyze C26 hydroxylations of cholesterol and cholest-4-en-3-one [367]. However, the *M. smegmatis* P450s have approximately twofold lower steroid substrate

affinity than the Mtb enzymes. Similar to Mtb, CYP142A2 can also compensate for CYP125A3. However, neither *CYP125A3* nor *CYP142A2* is essential in *M. smegmatis*. This was demonstrated through construction of a *ΔCYP125A3, ΔCYP142A2* double knockout mutant that maintains its ability to grow on cholesterol, with no build-up of cholest-4-en-3-one (that is toxic to Mtb). This indicates there is an additional level of redundancy in *M. smegmatis* that may involve another P450 enzyme, or even an alternative cholesterol catabolic process [367]. *M. smegmatis* possesses 39 P450 enzymes (in contrast to 20 in Mtb), and there are three putative CYP125 enzymes, with CYP125A4 and CYP125A5 identified in addition to CYP125A3. CYP125A5 is C-terminally truncated in comparison to the other CYP125 enzymes, but still retains the cysteine ligand and the heme-binding and EXXR motifs, and so it is unclear whether *M. smegmatis CYP125A5* is a pseudogene or encodes a functional enzyme [19]. Furthermore, *CYP189A1* is induced at low levels in *M. smegmatis* strains grown on cholesterol, hinting at a role for this P450 in cholesterol catabolism [367]. The CYP125A5 and CYP189A1 enzymes are as yet uncharacterized, but it is likely that they account for the *M. smegmatis ΔCYP125A3, ΔCYP142A2* double mutant's ability to grow on cholesterol. The structures of CYP125A3 and CYP142A2 have been determined and are similar to their Mtb orthologs, with some deviations in a portion of the substrate-binding region [360, 362, 363, 410]. The structure of CYP125A3 was determined in the ligand-free form (PDB 4APY) and is highly similar to the Mtb CYP125A1 structure [367]. These P450s share 77% amino acid sequence identity and the major differences between the two structures are seen with the presence of bulky residues Trp83, Met87, and Leu94 situated in the lower portion of the *M. smegmatis* CYP125A3 substrate access channel, compared with Phe100, Ile104, and Val111 in Mtb CYP125A1. The structure of CYP142A2 was determined in the ligand-free and cholest-4-en-3-one substrate-bound forms (PDB 3ZBY and 2YOO, respectively) [367]. Comparisons of the *M. smegmatis* and Mtb CYP142 structures reveal more diversity between the orthologs from these organisms than is observed for the CYP125 structures. The CYP142A1 and CYP142A2 enzymes have a similar level (78%) of amino acid sequence identity as was seen for the CYP125 orthologs. The CYP142A3 ligand-free structure was found to contain cyclodextrin, the carrier molecule used to solubilize cholesterol substrates. However, cholest-4-en-3-one substrate was not present in this structure. The cholest-4-en-3-one/CYP142A3 structure shows the G-helix interacting with the substrate and closing the active site cavity. The cholest-4-en-3-one substrate is also bound in a conformation that disfavors C26 hydroxylation, as it is sterically constrained by amino acid side chains in the substrate access channel, and oriented away from active site catalytic residues. Similar to CYP125A3, CYP142A3 also contains bulkier amino acids that interact with the base of the tetracyclic steroid ring and the top of the aliphatic side chain of cholest-4-en-3-one. These *M. smegmatis* CYP142A2 residues Met75, Phe77, and Phe255 replace Leu72, Met74, Met222 in the Mtb CYP142A1 structure. It remains unclear whether these substitutions influence functions of these enzymes or their ability to bind different steroids. *M. smegmatis* is a soil bacterium that may encounter a range of environmental sterols, such as plant phytosterols. In contrast, Mtb derives its cholesterol substrate from the human host immune cells. It is thus possible that these enzymes have evolved divergently to facilitate oxidation of the specific types of steroid substrates encountered during host infection (Mtb) or growth in soil (*M. smegmatis*) [367].

CYP164A2 is a further P450 from *M. smegmatis* that has been structurally characterized [412]. The CYP164 family members are found in a small group of actinomycetes, including a few nonpathogenic and pathogenic mycobacteria, and in *S. peucetis* [19]. Interestingly, the CYP164 family also contains the sole P450 from *M. leprae* (CYP164A1), the etiological agent of leprosy. *M. leprae* is a curious mycobacterial pathogen that operates on a minimal gene set and possesses only approximately 40% of the genome of Mtb [69, 413]. CYP164A1 and CYP164A2 share 60% amino acid sequence iden-

tity. CYP164A2 binds fatty acids with a preference for the unsaturated (C18:2) linoleic acid, although the physiological substrate(s) in *M. smegmatis* are unknown. The structure of CYP164A2 was determined in the ligand-free (PDB 3R9B) and substrate-bound (3R9C) forms [412]. CYP164A2 has a large active site channel that can accommodate two molecules of econazole in the inhibitor-bound complex structure, with econazole binding accompanied by structural reordering and rearrangement of the BC loop to close the active site cavity. One econazole molecule is observed to coordinate directly to the heme iron, whilst the second molecule binds in a pocket formed by the amino acid side chains of the C-helix [412]. Further characterization of this enzyme will be required to provide insights into the properties of CYP164A2 and of the *M. leprae* ortholog CYP164A1. It is important to understand the function of the only remaining P450 in *M. leprae,* and analysis of CYP164A1 should reveal a crucial role in the bacterium to explain why the pathogen has retained only this particular P450 enzyme during the massive decay of its genome that has occurred during its evolution [69].

CYP121A1 is an essential Mtb P450 that produces the metabolite mycocyclosin, via the oxidative biaryl coupling of its cyclo-*L*-tyrosine-*L*-tyrosine (cYY) substrate (Fig. 6.22a). The cYY is in turn produced by the genetically adjacent CDP synthase that binds and cyclizes two molecules of *L*-Tyr bound to the enzyme as amino acyl transfer RNAs (tRNAs) [65]. The CYP121A1 crystal structure has been solved at very high resolution (1.06 Å), enabling novel insights into elements of general P450 structure. The structure of CYP121A1 was determined in the ligand-free (PDB 1N40) [187], fluconazole inhibitor-bound (PDB 2IJ7) [414], and cYY substrate-bound forms (PDB 3G5H) [65] (Fig. 6.24c). Structures of CYP121A1 active site mutants (e.g., PDB 3CXZ and 3CY0) have also been determined [350], as have complexes of CYP121 with cYY analogs bound (PDB 4ICT and 4IPW) [415]. The high-resolution CYP121 structure revealed interesting P450 structural features, such as the presence of the heme in two distinct orientations, related by a 180° 'flip' of the cofactor. Furthermore, the heme was observed to be kinked at one of the pyrrole rings rather than adopting a planar structure. The large active site of CYP121A1 is constrained by a hydrogen-bonding network of amino acid side chains and water molecules, and amino acids Arg386 and Ser237 were implicated as likely participants in a proton delivery pathway for catalysis [187, 350]. An extended network of hydrogen bonds were identified that led from the protein surface to the active site, identifying a clear route that would allow proton transfer to iron–oxo intermediates in the CYP121A1 catalytic cycle, as well as the replenishment of the protons from bulk solvent [187]. The fluconazole-bound CYP121 structure also revealed a novel mode of inhibitor binding, showing that coordination of the heme iron occurs directly via a fluconazole triazole nitrogen (~30% of the ligated molecules in the crystal), but also indirectly in a mode by which the triazole nitrogen bridges to the heme iron via the interstitial sixth water ligand that remains on the heme iron (~70%). These findings were consistent with data collected for fluconazole binding using UV-visible and electron paramagnetic resonance (EPR) spectroscopic methods [414]. CYP121 binds azole drugs extremely tightly (some azoles have nanomolar dissociation constants), particularly in the case of the imidazole derivatives (e.g., econazole) rather than some of the newer, more water-soluble triazole drugs (e.g., fluconazole). The CYP121 K_d values for a number of azole compounds mirror their minimal inhibitory concentration (MIC) potency profiles against Mtb, suggesting that CYP121A1 is a likely target for these drugs [416]. Moreover, the imidazole-derived azoles clotrimazole and econazole were shown to be effective against both persistent and multidrug-resistant strains of Mtb [417, 418]. Econazole and clotrimazole were also shown to exhibit synergistic antimycobacterial activity when applied in combination with either of the commonly used front line anti-TB drugs rifampicin and isoniazid [419], and econazole was able to dramatically reduce the Mtb burden in the lungs and spleen of infected mice [417]. The use of azole drugs as agents to target CYP121A1 (and other Mtb P450s) is a tempting prospect. However, it may

be challenging to engineer these azole drugs to be more effectively tolerated orally, whilst still retaining their potency against Mtb. The CYP121A1 substrate-bound structure revealed cYY in the active site with one of the tyrosyl moieties pointing into the heme plane and hydrogen bonding with key active site residues [65] (Fig. 6.24c). This cYY-bound structure allowed the proposal of a model for the mechanism of C–C coupling to form mycocyclosin [65]. Substrate analogs have been utilized to explore the substrate specificity of CYP121A1 and to confirm its preference for cYY over other cyclic dipeptides, leading to the design of potential inhibitors specific for CYP121A1 [415]. In addition, the high-resolution crystal structures determinable for CYP121A1 and its complexes have facilitated the application of fragment-based screening and drug design studies, and these approaches have been successful in producing a CYP121A1 inhibitory scaffold that shows reasonable enzyme inhibition profiles at this preliminary stage of its development [420]. Further development and testing of such inhibitor molecules will be required in order to reveal their antibacterial potency and their potential in the development of CYP121A1 as a viable anti-TB drug target.

CYP130A1 is an orphan Mtb P450 with no known substrate or catalytic role to date [409]. It is one of the two P450s (including CYP141A1) whose genes are absent from the Mtb vaccine strain *M. bovis* BCG. *CYP130A1* and *CYP141A1* are among the genes found in the various *M. bovis* BCG genomic 'regions of deletion' (RDs). These RDs are considered to contain key genes responsible for virulence and have been mapped onto the Mtb genome. *CYP130A1* is located in the RD13 (RD10 in Behr's nomenclature) with *CYP141A1* in RD12 (RD05) [421, 422]. In addition, the RD regions containing *CYP130A1* and *CYP141A1* are also absent from the virulent *M. bovis* strain [421], and it is postulated that while these regions are not essential for bacterial growth per se, they may play a role in the infectivity of Mtb towards the human host [423, 424]. The CYP130 family is present in mycobacteria and in certain *Rhodococcus* spp. *CYP130A1* (*Rv1256c*) is chromosomally adjacent to a TetR-like transcription factor (*Rv1255c*) and a putative flavin adenine dinucleotide (FAD)-containing lactate dehydrogenase (*Rv1257c*). Despite its genetic localization giving little clue to the function of the CYP130A1 enzyme, this genomic organization is retained in the *CYP130* ortholog chromosomes. CYP130A1 has been structurally characterized in the ligand-free (PDB 2UUQ) and econazole-bound (PDDB 2UVN) forms. The ligand-free P450 crystallizes as a monomer in an open conformation. In contrast, the econazole-bound CYP130A1 crystallizes as a dimer in a closed conformation, with an extensive dimerization interface. The transition between the open and closed structures reveals a repositioning of the BC loop and FG helices, and econazole coordinates directly to the heme iron via its imidazole nitrogen, making additional hydrophobic interactions within the active site cavity. Solution-state-binding studies reveal that econazole displays apparent cooperative binding to CYP130A1. This may arise from effects of econazole on the interactions between CYP130A1 monomers, potentially promoting CYP130 dimerization in the ligand-bound state [409]. High-throughput compound screening studies identified a number of type II ligands, predominantly heterocyclic arylamines, and crystal structures reveal ligation of the molecules to the heme iron via nitrogen atoms [108]. In the absence of a substrate or catalytic role, it is presently difficult to ascertain what role CYP130A1 plays in Mtb. Routes to defining functions for such an 'orphan' P450 might include metabolic profiling studies, involving analysis of the differences in the metabolomes of wild-type (WT) and *ΔCYP130A1* Mtb strains. This could allow the identification of novel metabolites or compounds 'missing' in the gene deletion strain, or might pinpoint other changes in the metabolome that could provide clues as to how the P450 gene deletion impacts on known metabolic pathways.

The final Mtb P450 to be discussed is CYP51B1, the first Mtb P450 to be characterized. The discovery of a prokaryotic CYP51 [11, 425] was an exciting discovery in the P450 field and changed the perspective that *CYP51* genes were confined to eukaryotes. It is now clear that the

sterol demethylase CYP51 enzymes are conserved across all phyla [426]. Bacterial CYP51B family members have been found in other mycobacteria and actinomycetes, such as *Streptomyces* spp. and *Rhodococcus* spp. [19]. CYP51 enzymes catalyze the 14α-demethylation of sterol substrates (e.g., lanosterol, dihydrolanosterol, and obtusifoliol) to produce demethylated sterols that are key components of membrane integrity [427] (Fig. 6.25). Fungal CYP51 enzymes (particularly in *Candida albicans* and various *Aspergillus* spp.) are the targets of azole drugs, with inhibition of the sterol demethylases leading to disruption of membrane structure. A range of different azole scaffolds have been developed and many of these have seen successful applications in human and veterinary medicines, as well as in agriculture [428]. The identification of CYP51B1 in Mtb posed the obvious question as to whether the azole drugs would have activity against CYP51B1 and be effective against Mtb, particularly at a time when drug resistance in Mtb has become a major problem [429]. CYP51B1 was shown to be a bona fide sterol demethylase that can catalyze oxidative demethylation of lanosterol, dihydrolanosterol, and (most effectively) the plant sterol obtusifoliol [22, 430] (Fig. 6.25). However, in contrast to its eukaryotic counterparts, *CYP51B1* is nonessential to Mtb [348, 431]. Its role in Mtb thus remains obscure and sterols are apparently not found in Mtb membranes. As discussed above, the azole drugs are effective in inhibiting Mtb growth, and may target a number of different Mtb P450 enzymes. CYP121A1 is one of the likely candidates, in view of its gene essentiality and its high affinity for a number of the azoles that are most effective against Mtb [416]. Various azoles also bind avidly to CYP51B1 (though not as tightly as they do to CYP121A1), but in view of lack of evidence for *CYP51B1* gene essentiality in vitro or in vivo it appears unlikely that CYP51B1 is a major target for azole drugs. However, it is possible that CYP51B1 has the ability to utilize host sterols as substrates and to modify (likely demethylate) them for other purposes within Mtb, as described for the cholesterol oxidase P450s above. However, at present the true physiological role of Mtb CYP51B1 remains unknown. The eukaryotic CYP51's are membrane bound and the identification of CYP51B1 as a soluble bacterial P450 was thus of great interest. The soluble nature of CYP51B1 facilitated its crystallization and the structural characterization of the first CYP51 P450 enzyme from Mtb, and thus led CYP51B1 to become a model enzyme for its eukaryotic counterparts [408, 432, 433]. Detailed structural and mechanistic studies have since been done on CYP51B1 (e.g., [434, 435]). The structure of CYP51B1 was solved in the ligand-free form (PDB 2BZ9) [436], and in forms bound to the inhibitor fluconazole (PDB 1EA1) [408] and to the substrate analog estriol (PDB 1X8V) [437]. The overall structures of CYP51B1 revealed a deviation in the I-helix that results in a large kink [408] compared to various other P450 structures [24, 112]. This I-helix kink is structurally perturbed upon ligand binding, enabling conformational changes that allow CYP51B1 to adapt to different sized ligands [408, 438]. Structural analysis of CYP51B1 also defined two distinct channels that suggest discrete sites of substrate/product entry and exit through movement of the BC (particularly between the B′ and C helical sections of the P450) and FG regions [408, 437]. The first channel is similar to the conventional FG loop substrate entry regions that are perpendicular to the plane of the heme, as described for other P450s such as BM3 (e.g., [24, 271]). The second channel is roughly parallel to the heme plane and is formed by the BC loop region, creating a chamber at the junction of the B′ and I helices, β strands β1–4 and β4–1,2, and the loop connecting the K-helix and β strand β1–4 [408]. *CYP51B1* (*Rv0764c*) is adjacent on the Mtb chromosome to a ferredoxin gene (*Rv0763c*) that was shown to encode a 3Fe–4S ferredoxin (Fdx) that can supply electrons to the P450 (when partnered with a heterologous NAD(P)H-dependent flavoprotein reductase, or the Mtb FprA reductase), and that is a likely endogenous redox partner for CYP51B1 [21, 430]. CYP51B1 undergoes rapid conversion from P450 to P420 in its ferrous–CO complex [425]. Anaerobic UV-visible spectral analysis of the reduction of CYP51B1 indicated that protonation of the cysteine thiolate occurred even

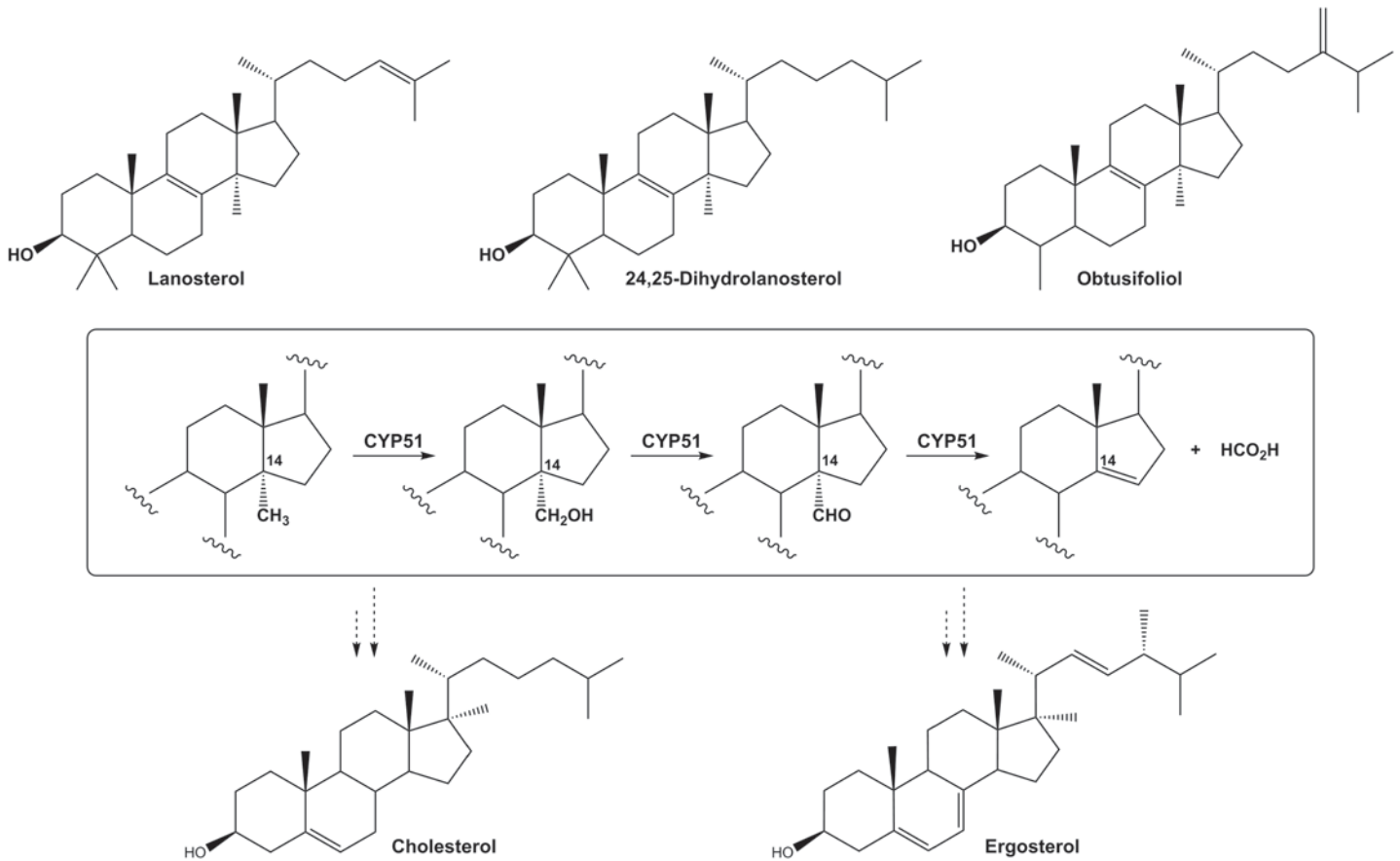

Fig. 6.25 The major CYP51 substrates lanosterol, 24,25-dihydrolanosterol, and obtusifoliol. The *upper* panel shows the structures of CYP51 substrates lanosterol and 24,25-dihydrolanosterol (substrates for mammalian and yeast CYP51s), and of the plant (and trypanosome) sterol substrate obtusifoliol [21, 447]. The *central* panel shows the CYP51 sterol demethylation reaction that occurs by three consecutive oxidative reactions on the sterol 14α-methyl group to generate alcohol, then aldehyde, and then demethylation with release of formate. The lanosterol and 24,25-dihydrolanosterol demethylation products ultimately go on to form ergosterol and/or cholesterol. The obtusifoliol-demethylated product can ultimately be converted to sitosterol, or to other sterols including ergosterol. Cholesterol has an important role in Mtb infection and can be oxidized by Mtb P450s [356–358, 851], while ergosterol is the major membrane sterol in yeast [446, 852]

in the absence of the gaseous ligand, indicating instability of the thiolate ligand to heme iron reduction. The rate constant for cysteine thiolate protonation decreased on reduction of the estriol complex of CYP51B1, pointing to an important role for a physiological substrate in stabilizing the active form of CYP51B1 [21]. Compound screening studies identified both type I and type II ligands with affinity for CYP51B1 [436]. The leading type I ligand DHBP (4,4′-dihydroxybenzophenone) was crystallized in its complex with CYP51B1, and provided the most complete CYP51B1 structure to date. In preceding crystal structures, various parts of the CYP51B1 structure were seen to be flexible/disordered. A structural 'ordering' was observed in the CYP51B1–DHBP complex, particularly in the BC loop region, and the structural arrangement of the CYP51B1–DHBP complex mimicked the substrate-bound conformation of the P450 [438], similar to the estriol substrate analog-bound CYP51B1 crystal structure [437]. The type II ligands EPBA (α-ethyl-*N*-4-pyridinyl-benzeneacetamide) and the related BSPPA ((2-(benzo[d]-2,1,3-thiadiazole-4-sulfonyl)-2-amino-2-phenyl-N-(pyridinyl-4)-acetamide) also produced a degree of structural reordering, and crystal structures with these compounds demonstrated direct coordination of the ligand pyridine group to the heme iron [436]. Interestingly, EPBA was also shown to be inhibitory to the growth of Mtb [436]. A common *N*-(4-pyridyl)-formamide moiety was used to develop second-generation molecules for CYP51B1 structural characterization [439]. Further elaboration of these scaffolds may lead to CYP51B1-specific inhibitors. In addition, these compounds could provide useful tools to study the effects of CYP51B1 inhibition on Mtb (e.g., using transcriptomics or metabolomics) in efforts to elucidate the P450's physiological role. A number of the second-generation compounds were also shown to possess a greater affinity for the CYP51E sterol demethylase from *Trypanosoma cruzi* than for CYP51B1 [439]. *T. cruzi* is a parasitic protozoan pathogen that is the causative agent of Chagas disease [440]. A CYP51B1-derived molecule (α-[[(4-methylcyclohexyl) carbonyl]amino]-*N*-4-pyridinyl-1H-indole-3-propanamide) displayed selective and potent activity against *T. cruzi* in an infected mouse macrophage model [439].

The Mtb P450s thus constitute an interesting group of enzymes with considerable diversity in substrate specificity and catalytic roles, spanning functions including secondary metabolism, respiratory regulation and the catabolism of steroids. Many of these P450 remain uncharacterized and the determination of their properties will presumably reveal further unexpected oxidative functions in this remarkable bacterial pathogen. The Mtb P450 cohort encompasses many exclusive P450 families, as well as containing an evolutionarily widely conserved P450 in the case of CYP51B1. It seems likely that Mtb has selectively assimilated and retained the *CYP51B1* gene during its evolution as a human pathogen, but any key role in host–pathogen interactions remains obscure. It may be the case that possessing an enzyme with a human-like ability to demethylate or otherwise modify host sterols is an important part of Mtb's strategy for infection and survival in the human host. However, much research still remains to be done to define the main catalytic roles of CYP51B1 and several other Mtb P450s. Comparisons of all the Mtb P450 structures, depicted as an overlay of their carbon backbones (Fig. 6.26), help to demonstrate the diversity of their secondary structural elements, while also illustrating the retention of the overall tertiary structural fold that defines the P450 enzyme class. The observed structural differences depict diverse protein conformations which, although often appearing as subtle movements of the P450 secondary structural elements, can lead to extensive variability of P450 structure and substrate selectivity (in terms of size, shape, and chemical character). These dynamic conformational changes in P450 structures are required to allow the conserved P450-specific fold to adapt to a wide variety of substrates, to catalyze a range of oxidative reactions and to bind productively to substrate delivery systems (e.g., PCP and ACP accessory proteins) and to diverse redox partners.

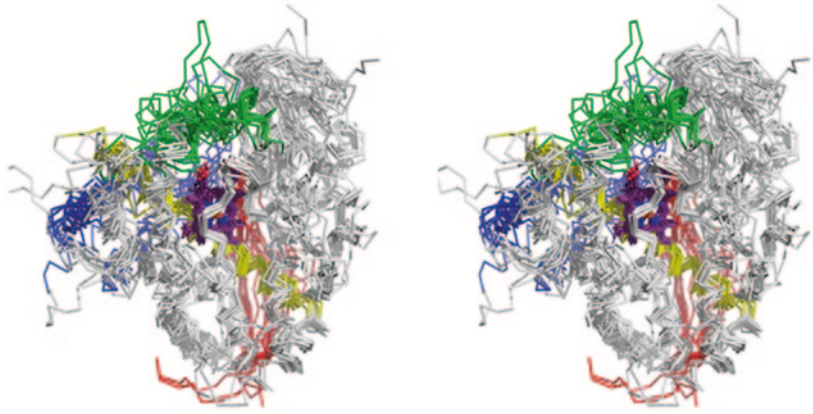

Fig. 6.26 Multiple overlay of bacterial cytochrome P450 structures. An overlay of bacterial P450 structures reveals large variability. A stereo-view is presented for the structural alignments of all the P450 enzyme structures from *Mycobacterium tuberculosis* present in the database, together with those of the three model microbial P450s shown in Fig. 6.2 (P450s EryF, BM3, and Cam). Color coding of the structural elements is the same as in Fig. 6.1. Among the few regions that remain largely invariant across all the P450s is that containing the cysteine proximal ligand to the heme iron, along with the central I-helix region (*yellow*) flanking the heme. The largest variations are observed in the main substrate-binding elements (i.e., the BC loop in *green* and FG helices in *blue*)

6.2.4 P450s in Fungi

Fungi are a large and varied group encompassing lower eukaryotic microorganisms such as yeasts, molds, and basidiomycetes, with more than a million species known. They play important roles including the cycling of elements in the biosphere and the degradation of toxic environmental pollutants [441]. Several databases have been established to support the systematic classification of fungal P450s arising from the large number of genomes already sequenced, and to accommodate data from ongoing and future fungal genome sequencing projects [19, 165, 442, 443]. Analyses of the genomes of fungal organisms have revealed large numbers of P450 genes. Although most of these organisms have fewer P450s than found in plants and insects [19], fungal P450s still show an enormous diversity of form and function, and serve numerous important physiological and ecological roles, being particularly important in recycling of nutrients through breaking down a wide range of organic materials [77]. At the time of preparation of this manuscript, there are 8731 P450 genes across 113 different families identified in the genomes of fungal and oomycete species that are present in the Fungal Cytochrome P450 Database (FCPD) [443]. Of these fungal genes, 6418 have been classified and annotated by David Nelson [19].

Fungi can inhabit diverse ecological niches and some of the fungal P450 enzymes were shown to contribute to fungal survival in such niches. The fungal P450s also possess a wide variety of functions across diverse enzyme families [77]. Across the entire fungal kingdom, only the CYP51 and CYP61 family P450s are consistently conserved, with these enzymes having essential functions in the synthesis of the membrane sterol ergosterol [77, 444–447] (Fig. 6.25). Fungi can have quite different numbers of P450s. *Aspergillus oryzae* has 155 *CYP* genes (the number including 13 pseudogenes), while the pathogen *A. fumigatus* has only 74 [15, 448, 449]. The larger number of *CYP* genes in *A. oryzae* reflects gene duplication and lateral gene transfer events during the evolution of this organism [450]. However, not all fungal organisms contain large numbers of *CYP* genes. For example, the fission yeasts from *Schizosaccharomyces* spp., including *S. japonicus, S. pombe,* and *S. octosporus*, contain only the two CYP51 and CYP61 housekeeping genes [19, 451]. Fungal P450s possess catalytic activities that are essential to many primary

and secondary metabolite synthetic pathways, in addition to their roles in production of ergosterol [446, 452–454] (summarized in Table 6.5). These include diverse roles in the generation of cell wall components [455–457]; in signaling factor biosynthesis [458, 459]; in the production of mycotoxins/phytotoxins (e.g., aflatoxins, fumonisins, and trichothecenes); and in synthesis of gibberellin in endophytic fungi [460–464]. Further, important roles of fungal P450s involve the degradation of environmental pollutants, including P450-mediated oxidation of endocrine disrupting agents (alkylphenols) and of recalcitrant polycyclic aromatic hydrocarbons (PAHs) [76, 465, 466].

6.2.4.1 The First Membrane-Bound P450 Structure

A major breakthrough in the P450 field was the recent publication by Monk et al. that describes the structure of the sterol demethylase CYP51F1 from *Saccharomyces cerevisiae* [467]. CYP51F1 is a membrane-bound lanosterol 14α-demethylase that catalyzes the first step in ergosterol biosynthesis [468, 469] (Fig. 6.25). This enzyme is a major drug target and is inhibited by several azole drugs that bind tightly to the enzyme and use their imidazole/triazole groups to coordinate to its heme iron [426, 470]. The CYP51F1 structure is the first full-length P450 crystal structure that includes the N-terminal membrane-spanning region. This region is absent in the soluble prokaryotic and archaeal P450s, and is usually truncated to ease the handling of membranous P450 proteins during in vitro studies, and to help facilitate their crystallization [471]. Many P450 substrates, particularly in the eukaryotic enzymes, are hydrophobic molecules such as sterols, fatty acids, and lipophilic drugs. These are likely delivered to the relevant P450 enzyme via the lipid bilayer [472]. The structure of CYP51F1 (Fig. 6.27) displays a clearly defined and well-ordered transmembrane helical domain that links CYP51F1 to an N-terminal amphipathic helix that forms extensive contacts with other CYP51F1 molecules within the crystal asymmetric unit. The amphipathic N-terminal helix, the transmembrane region, and the core CYP51F1 structure sit as three distinct domains. The structural organization of the N-terminal helix and its distribution of hydrophobic/hydrophilic residues suggest that the hydrophobic face of this helix may lie naturally along the inner side of the endoplasmic reticulum membrane. At the other side of the membrane, polar interactions help to constrain the orientation of the catalytic (P450) domain and to position it such that portions of the loop region between the F–F′ and G helices become buried in the membrane. These data are consistent with preceding experiments that indicate that this part of the P450 structure is important for membrane association [472–474]. It is proposed that a cluster of charged residues at the N-terminal of the G-helix interact with phosphate head groups in the lipid bilayer, placing the substrate-binding channel of the P450 at the cytoplasmic surface of the bilayer to enable access to the membrane-associated sterol substrate [467]. The lanosterol-bound CYP51F1 structure shows electron density that locates the position of the lanosterol in the active site cavity (although it is not completely defined), with the P450 heme iron likely in the ferrous–oxy form, presumably as a result of X-ray-mediated heme iron reduction. In addition, there is electron density observed that is consistent with a second lanosterol-sized molecule located in a secondary binding site, and this may identify a substrate-sampling mode or a product exit channel. Mapping of drug resistance-associated amino acid substitutions from azole-resistant clinical isolates of *Candida* spp., *Aspergillus* spp., *Cryptococcus neoformans,* and *Ajellomyces capsulatus* reveals the locations of these mutations in the fungal CYP51F1 structure. For instance, the commonly occurring azole resistance mutation, Y132F/H in *C. albicans* (Y140 in CYP51F1) [475, 476], may exert its effect (at least in part) due to the loss of a heme-binding hydrogen bond that could modify the heme orientation and diminish susceptibility towards azole binding [467]. The CYP51F1 structure represents an exciting development in the structural biology of P450s and is a major step forward in our understanding of membrane interactions and their effects on P450 structures. The data provide new insights into CYP51 structure/function and

Table 6.5 Functionally characterized fungal P450s

P450	Reaction	Functional role	Organism(s)	Refs
CYP51	14α-sterol demethylation	Biosynthesis of membrane ergosterol	E.g., *C. albicans, S. cerevisiae*	E.g., [452]
CYP61	C22-sterol desaturation	Biosynthesis of membrane ergosterol	E.g., *C. albicans, S. cerevisiae*	E.g., [453]
CYP52 (P450Alk)	ω-hydroxylation of n-alkanes and fatty acids	Degradation of n-alkanes and fatty acids as carbon sources for energy	*Candida* spp., *Yarrowia lipolytica, Starmerella bombicola, Beauveria bassiana*	[509, 820–826]
CYP53	Benzoic acid (and derivatives) hydroxylation and trimethoxy-trans-stilbene O-demethylation	Benzoate and its derivatives degradation/detoxification and synthesis of stilbene derivatives	*A. niger, A. nidulans, Cochliobolus lunatus, P. chrysosporium* and *Postia placenta*	[502, 827–830]
CYP55	Nitric oxide reductase	Denitrification	*F. oxysporum, Cylindrocarpon tonkinese, A. oryzae, T. cutaneum*	[516, 611, 613, 831]
CYP56	C–C coupling of N-formyl tyrosine	Formation of N, N′-bisformyl dityrosine for outer spore wall production	E.g., *C. albicans, S. cerevisiae*	[455–457, 832]
YP57	6a-pisatin demethylation	Pisatin detoxification	*N. haematococca, F. oxysporum*	[833–835]
CYP58	Epoxidation of o-methyl-sterigmatocystin precursor and trichodiene C-2 hydroxylation, C12–C13 epoxidation, C11 and C3 hydroxylations	Aflatoxin and trichothecene biosynthesis	*A. flavus, A. parasiticus*; and *F. graminearum, F. sporotrichoides*	[512, 836] [513, 514]
CYP59	Norsolorinic acid/averantin hydroxylation	Aflatoxin biosynthesis	*A. flavus, A. parasiticus*	[512, 836] [513, 741, 837]
CYP60	Versicolorin B hydroxylation and desaturation			
CYP62	Not known	Aflatoxin biosynthesis	*A. flavus, A. parasiticus*	[512, 836] [513, 838]
CYP63	Polycyclic aromatic hydrocarbon, alkylphenol, and alkane hydroxylation	Detoxification of xenobiotics and industrial alkane assimilation	*P. chrysosporium, Phlebia brevispora*	[465, 839]
CYP64	Hydroxylation of o-methylsterigmatocystin	Aflatoxin biosynthesis	*A. flavus, A. parasiticus*	[512, 836] [513, 838]
CYP65	Trichothecene C15 hydroxylation and fumonisin C10 hydroxylation	Trichothecene biosynthesis and fumonisin biosynthesis	*F. graminearum, F. sporotrichoides* and *F. verticillioides*	[514, 840, 841]
CYP68	Trichothecene C8 hydroxylation and GA14 and GA12 hydroxylations	Trichothecene biosynthesis and gibberellin biosynthesis	*F. graminearum, F. sporotrichoides* and *F. fujikori*	[462, 514, 842, 843]
CYP69	GA4 and GA7 C13 hydroxylations	Gibberellin biosynthesis	*F. fujikori*	[462, 842–844]
CYP503	ent-Kaurene hydroxylations (x3)			

Table 6.5 (continued)

P450	Reaction	Functional role	Organism(s)	Refs
CYP504	Phenylacetate C2 hydroxylation, 3-hydroxy- and 3,4-dihydroxyphenyl-acetate C6 hydroxylations	Degradation of phenylacetate and its derivatives	*A. nidulans*	[518, 519]
CYP505	ω-1 to ω-3 fatty acid hydroxylations (P450foxy) and fumonisin C14 and C15 hydroxylations	Fatty acid degradation and fumonisin biosynthesis	*F. oxysporum* and *F. verticillioides*	[504, 693, 694] [840, 841]
CYP526	Trichothecene C4 hydroxylation	Trichothecene biosynthesis	*F. graminearum, F. sporotrichoides*	[514]
CYP5136	Polycyclic aromatic hydrocarbon and alkylphenol hydroxylations	Detoxification of xenobiotics	*P. chrysosporium*	[466]
CYP5138	Naringenin 3′-hydroxylation and dibenzo-p-dioxin, 2-monochloroDD, biphenyl and naphthalene oxidation	Flavinoid biosynthesis and pollutant degradation	*P. chrysosporium*	[845]
CYP5147	Flavone 3′-hydroxylation and 7-eth-oxycoumarin O-dealkylation	Flavinoid biosynthesis and potential pol-lutant degradation	*P. chrysosporium*	[846]
CYP6001	Linoleate 5,8-diol synthase and 10R-linoleate dioxygenase	Oxylipin psi-factor biosynthesis (Ppo P450s)	*A. clavatus, A. fumigatus, A. flavus, A. nidu-lans, A. niger*	[459, 480, 487–489, 498] [495]

GA gibberellin, *A* aspergillus, *C* candida, *F* fusarium, *N* haematococca—Nectria haematococca, *P* phanerochaete, *S* saccharomyces, *T* trichosporon

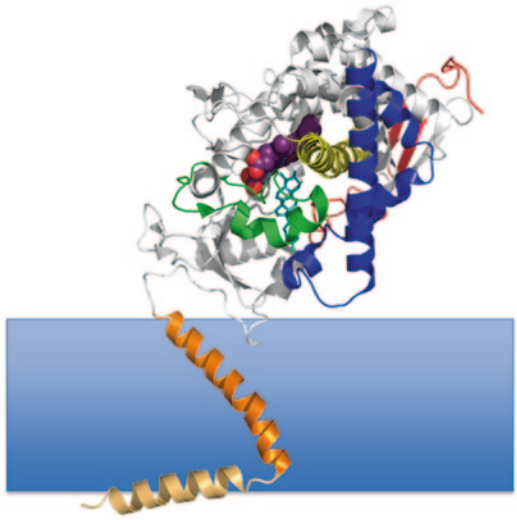

Fig. 6.27 The structure of a membrane-bound microbial P450. The crystal structure of the intact yeast (*S. cerevisiae*) CYP51F1–lanosterol complex is shown, with the transmembrane spanning N-terminal region in *dark orange* and the amphipathic N-terminal helix in *light orange*. The P450 is oriented such that the substrate-binding region faces the membrane (indicated by a *blue box*), so that the hydrophobic substrate can be accessed directly from the lipid bilayer [467]

could provide important information relevant to new antifungal drug development against a proven target P450 enzyme. Detailed descriptions of the role of fungal CYP51s, their inhibition by azole drugs and mutations that confer drug resistance are given in previous reviews [426, 447, 477].

6.2.4.2 The Ppo Enzymes

An intriguing and important family of fungal P450s are the Ppo proteins that are involved in the production of oxylipins. The Ppo's participate in the regulation of the sexual and asexual fungal developmental life cycles, particularly in sporulation, and via their production of fungal oxylipins. These are oxygenated metabolites of linoleic and oleic acids [478], and are termed precocious sexual inducers (psi factors) [459, 479]. The psi factors are also involved in formation of mycotoxins that are virulence factors for the fungal hosts [480]. Oxylipins are generally derived as products of lipid peroxidation reactions [481], and are ubiquitous hormone-like compounds shown to have pivotal roles as signaling molecules [482–485]. In plants and mammals, production of oxylipins is a multistep process that involves separate oxidation reactions, followed by isomerization of hydroperoxy intermediates by distinct proteins. The Ppo's are a unique enzyme class that has evolved to form a natural fusion of an N-terminal heme peroxygenase/dioxygenase, and a C-terminal isomerase P450 in a single polypeptide [479, 480, 486]. Three oxidase/isomerase Ppo enzymes (PpoA, PpoB, and PpoC) have been identified in *Aspergillus* spp. [480, 481, 487], and were shown to oxidize their unsaturated fatty acid substrates to 8*R*- and 10*R*-hydroperoxy intermediates, with the former undergoing a P450-mediated rearrangement to the 5,8-dihydroxy derivative [488, 489]. These reactions, catalyzed by a single fungal enzyme, resemble those in the typical oxylipin biosynthetic pathways described in plants and mammals [490, 491]. PpoA (CYP6001A1) from *A. nidulans* was the first enzyme of this class to be characterized, and was shown to catalyze oxidation of linoleic acid (18:2*n*−6) (by the dioxygenase domain) to (8*R*)-hydroperoxyoctadecadienoic acid ((8*R*)-HPODE), with subsequent isomerization to (5*S*,8*R*)-dihydroxy-9*Z*,12*Z*-octadecadienoic acid ((5*S*,8*R*)-DiHODE) catalyzed by the P450 domain [488]. The P450 domain catalyzes a molecular rearrangement reaction that needs no external reducing equivalents, similar to those performed by the plant CYP74A (AOS) and the mammalian CYP5A (thromboxane synthase) and CYP8A (prostacyclin synthase) P450s. These P450s use fatty acid peroxides to supply both the substrate and the oxygen activator in order to bypass the canonical P450 catalytic cycle and to form compound II [113, 181, 182, 492, 493] (Fig. 6.4). A similar reaction also occurs in the related 7,8-linoleate diol synthase from *Gaeumannomyces graminis* that generates 7,8-dihydroxy linoleic acids [494]. The second *A. nidulans* Ppo enzyme, PpoC (CYP6001C1), has 45 % amino acid sequence identity to PpoA and was found to be a linoleate 10*R*-dioxygenase, catalyzing oxidation of linoleic acid to the (10*R*)-hydroperoxyoctadecadienoic acid ((10*R*)-HPODE)

[17, 489]. Despite the presence of an apparent P450 domain, no isomerization activity could be shown. Amino acid sequence analysis and alignments revealed that the PpoC P450 domain does not retain the conserved P450 cysteine residue (with a glycine substituted at this position), and thus is not a functional P450 oxidase [17, 489]. The role of the third recognized Ppo enzyme (PpoB, CYP6001B1) is still unclear [489]. It was originally proposed that it may be involved in the metabolism of 8,11-DiHODE ((8*R*,11*S*)-dihydroxy-(9*Z*,12*Z*)-octadecadienoic acid), a product that was detected in the Ppo-containing *Aspergillus* sp. during linoleic acid oxygenation studies [487, 495]. However, gene disruption studies have shown that this metabolite is still produced in the absence of PpoB. It therefore appears that the 8,11-DiHODE synthase in this organism is distinct from PpoB [496]. The production of 8,11-DiHODE is repressed by common P450-inhibiting azole drugs, which may indicate that another unidentified P450 is involved in formation of this metabolite. It appears unlikely that PpoA is the P450 that also produces 8,11-DiHODE. However, in studies of individual *ppoA, ppoB* and *ppoC* insertion mutant strains of *A. fumigatus* strain AF293, it was found that while radial growth rates of the mutant and WT strains were similar, the *ppoC* mutant strain was affected in conidial development, germination, and oxidative stress tolerance, and also showed increased uptake and destruction by alveolar macrophages [497]. As is the case for PpoC, PpoB also has a nonfunctional P450 domain in which a serine residue replaces the conserved cysteine. The absence of the cysteine residue is also seen in PpoB proteins from other *Aspergillus* spp. and it may be that a nonfunctional Ppo P450 domain has been retained through evolution in Ppo's B and C for reasons other than catalytic activity, perhaps to preserve a structural fold crucial for the activity or specificity of the dioxygenase domain. Genes encoding Ppo orthologs have been found in a variety of *Aspergillus* spp. [480, 487, 488, 495, 498] and in other sequenced fungal genomes [443], and BLAST searches reveal the conservation of orthologous genes throughout the ascomycetes. The preservation of the Ppo enzymes and their orthologs suggests that the psi factors, and likely Ppo-derived metabolites, play pivotal roles in fungal signaling, growth, and development. They also influence the fungal virulence and ability to infect mammalian and plant hosts. Some further aspects of the biochemistry of the Ppo enzymes are described in the section 'Microbial P450-(redox) partner fusion enzymes'.

6.2.4.3 P450s in the White Rot Fungus Phanerochaete Chrysosporium

There is extensive interest in organisms able to degrade polymers such as cellulose and lignin in order to exploit these natural resources for production of useful chemicals. The white rot basidiomycete fungi are able to degrade all of the components of plant cell walls, and are thus much studied for their biotechnological potential in this area [499]. The white rot fungi are so named due to their ability to degrade lignin, leaving colorless cellulose. Key enzyme catalysts involved are extracellular laccases along with lignin and manganese peroxidases [499]. In contrast, the brown rot basidiomycetes break down hemicellulose and cellulose components of plant cell walls, but do not degrade the lignin polymer (although modifications such as demethylation and phenolic hydroxylations are observed) [499–501]. In comparison to the white rot fungi, relatively little is known about the cellular biology of brown rot basidiomycetes. However, they are considered to have evolved from the white rot basidiomycetes, and probably to have more sophisticated secondary metabolism in comparison to white rot fungi, likely requiring a larger cohort of P450 enzymes in most cases [79, 81, 499, 502]. The white rot basidiomycete *Phanerochaete chrysosporium* has become a model organism in this area, and was shown to have an extensive number of P450 enzymes (151 *CYP* genes, compared to ~250 in the brown rot fungus *Postia placenta*) [79, 80, 502, 503]. A single *CPR* gene as well as genes encoding cytochrome b_5 and b_5 reductase are also present in *P. chrysosporium* [503].

The P450s in *P. chrysosporium* have been classified into 16 distinct gene clusters, and have been grouped into 31 different gene families [77, 503]. These include a single *CYP51* sterol demethylase

family member, but also large numbers of CYP genes classified into the *CYP512* (15), *CYP5035* (13), and *CYP5144* (35) families [77]. There are also seven members of the CYP505 family, whose most prominent fungal member is P450foxy, a fatty acid hydroxylase P450–CPR fusion enzyme [504]. In efforts to define catalytic activities of the *P. chrysosporium* P450s, several *CYP* genes were co-expressed in *S. cerevisiae* with the host CPR and screened for activities against a wide range of organic compounds [74, 502]. Oxidative activities were identified towards PAHs such as fluorene (CYPs 5136A1, 5136A3, and 5150A2), dibenzothiophene (e.g., CYPs 502B1, 512G2, 5144A13, and 5147A3), biphenyl (e.g., 5136A1, 5138A1, 5144A10, and 5145A3), and naphthalene (e.g., CYPs 5036A3, 5136A1, 5141C1, and 5150A2) [74, 502] (Fig. 6.28). Findings here are consistent with the potential of *P. chrysosporium* P450s to degrade PAH environmental pollutants, and with the theory that the basidiomycete fungi have evolved a survival strategy that involves an array of oxidative enzymes that enable them to degrade and utilize several xenobiotic compounds in addition to plant-derived compounds that include lignin and its breakdown products (including phenolics) [77]. Among other useful activities in the *P. chrysosporium* P450s are oxidation of steroids. CYP512 family members from both *P. chrysosporium* and *P. placenta* were found to oxidize progesterone and testosterone. Moreover, the *P. chrysosporium* P450s CYP512N and CYP512P oxidize both testosterone and the dehydroabietic acid, which likely relates to the structural relationships between the steroid and abietane diterpenoids from plants [74, 502]. Various other *P. chrysosporium* P450s were shown to oxidize compounds such as 7-ethoxycoumarin, diclofenac, compactin, and naproxen [499]. CYP5150A2 was also expressed and purified from *E. coli,* and shown to bind 4-pentylbenzoic acid tightly. For this P450, activity was also demonstrated with cytochrome b_5 and b_5 reductase partners in the absence of CPR [499, 505, 506].

While relatively little remains known on the structure and physiological function of the *P. chrysosporium* P450s, it is clear that they offer an array of oxidative activities that enable the oxidative degradation of a vast number of compounds emanating both from breakdown of lignin, and otherwise occurring naturally in the soil. There is clearly great potential in identifying their scope of catalytic activities in order to better understand how lignin-degrading fungi process the plant metabolites, and with respect to future application of these P450 enzymes for biotechnologically useful catalytic transformations.

6.2.4.4 Other Fungal P450 Reactions of Physiological and Biotechnological Importance

There are numerous examples of yeast and fungal P450s with activities crucial to survival of the organisms, or with potential for exploitation in biotransformations. For greater detail, the reader is directed towards recent reviews in the area that cover in depth the functional, structural, and evolutionary properties of these P450s (e.g., [446, 451, 454]. In addition to the key role of CYP51 enzymes in sterol demethylation (detailed elsewhere in this chapter), the CYP61 P450 was characterized in *S. cerevisiae* and *Candida glabrata* as a Δ^{22}-desaturase that introduces a side-chain double bond in the process of ergosterol biosynthesis. CYP61 was also implicated in oxidative detoxification of benzo[a]pyrene through production of 3-hydroxy benzo[a]pyrene [445, 453, 507, 508]. The CYP52 family of P450s is found in *Candida* spp. (e.g., *C. tropicalis* and *C. lipolytica*) that assimilate alkanes, and these P450s catalyze a rate-limiting step in hydroxylation of n-alkanes and fatty acids—which are then further metabolized via the β-oxidation pathway (e.g., [509–511]. Other key functions include the involvement of CYP58 family P450s in the synthesis of aflatoxins (e.g., [512] Wen 2005 [513]), and of the P450s from the CYP58, 65, 68, and 526 families in synthesis of sesquiterpene trichothecene mycotoxins in organisms such as *Fusarium graminearum* and *F. sporotrichoides* [446, 514]. Targeting P450s involved in making such toxins is clearly an attractive route to prevent the formation of such toxins, and in this respect the multiple early oxidation steps catalyzed by the CYP58 enzyme in trichothecene biosynthesis make this P450 an obvious target [515].

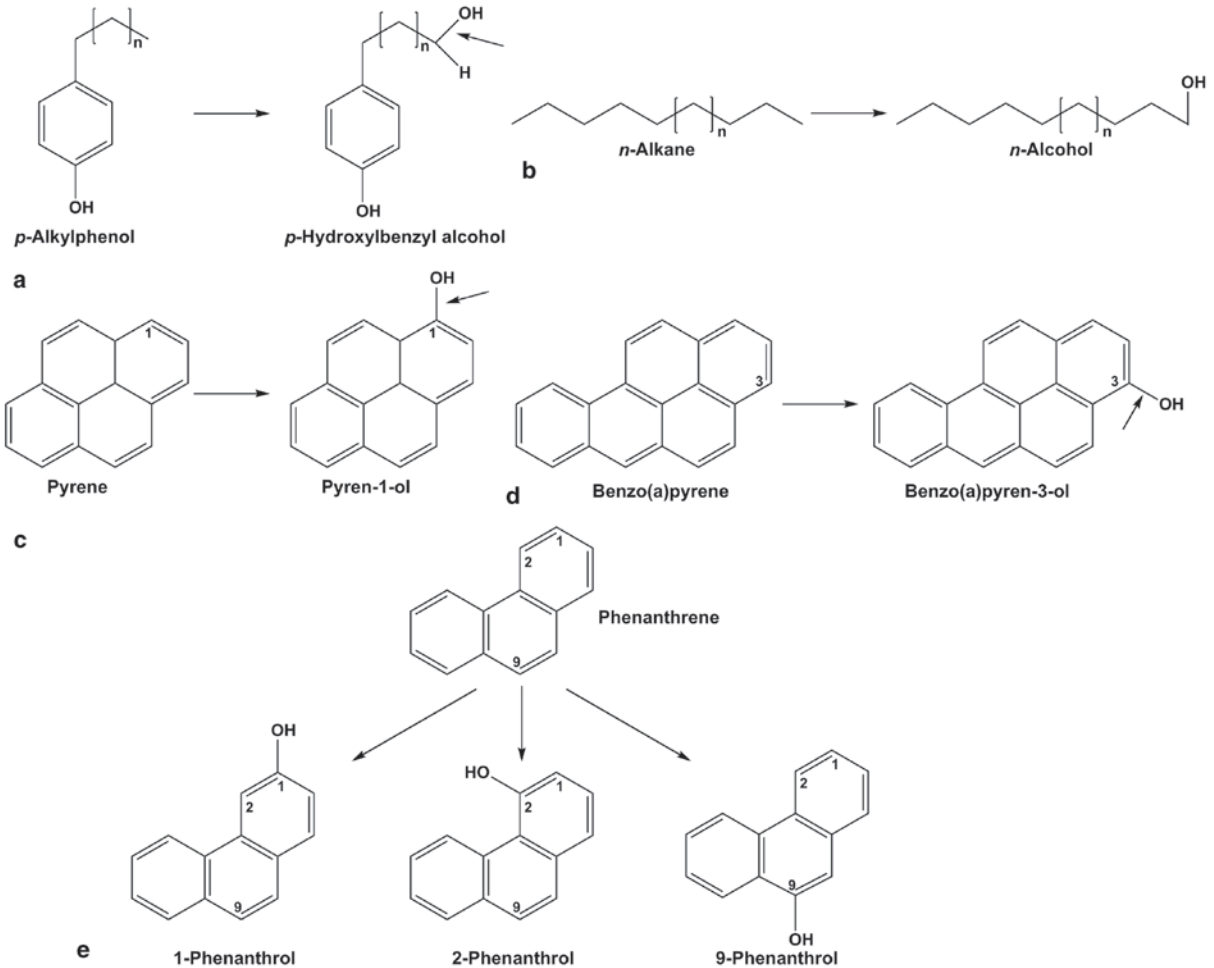

Fig. 6.28 Ligninolytic *white rot* fungi *Phanerochaete chrysosporium* P450 substrates and the diversity of CYP63A2 substrate selectivity. **a** The CYP63A2-catalyzed hydroxylation of endocrine-disrupting long-chain ($n = 1–7$) alkylphenols. **b** The hydroxylation of crude oil aliphatic hydrocarbon *n*-alkanes ($n = 1–4, 7–11$) by CYP63A2. The P450-mediated hydroxylations of the mutagenic/carcinogenic fused-ring higher molecular weight polycyclic aromatic hydrocarbons (PAHs) pyrene (**c**) by CYPs 63A2, 5136A2, 5136A3, 5142A3, 5144A5, 5144A7, and 5145A3; benzo(a)pyrene (**d**) by CYPs 63A2, 5136A2, and 5144A7; and phenanthrene (**e**) by CYPs 63A2, 5136A2, 5144A5, and 5145A3 [76, 465, 853]

Among other important roles for fungal P450s are the functions of CYP55 enzymes in denitrification through generation of dinitrogen oxide (N_2O) from two nitric oxide molecules in a reaction using only NAD(P)H and no exogenous redox partners (e.g., in *F. oxysporum* and *C. tonkinense*; see the section 'Fungal nitric oxide reductases') [516, 517], and the contributions of CYP504A1 and B1 in degradation of xenobiotic aromatic compounds—as shown in *A. nidulans* grown on phenylacetate and hydroxylated derivatives. CYP504A1 converts phenylacetate to 2-hydroxyphenylacetate, while CYP504B1 converts 3-hydroxyphenylacetate and 3,4-dihydroxyphenylacetate to homogentisate and 2,3,5-trihydroxyphenylacetate, respectively, leading to cellular metabolism of these molecules [518, 519].

It is clear that the repertoire of oxidative catalytic activities and number of P450s in yeasts and fungi is vast, and that there are obvious applications for several of these enzymes. To some extent, the membrane-bound nature of these P450s and their CPR partner has made their detailed structural and mechanistic analysis more challenging. However, recent breakthroughs including the development of nanodisk technology and the successful crystallization and structural elucidation of the *S. cerevisiae* CYP51 membrane protein point to future breakthroughs in our understanding of the structural organization of other yeast/fungal P450s [467, 520].

6.3 Redox Partner Systems and Their Diversity in Microbes

For many years, the type of redox protein systems that serve to pass NAD(P)H-derived electrons to cytochrome P450 enzymes were thought to be few—and limited mainly to two classes—essentially being 'bacterial' (class I) and 'eukaryotic' (class II) [521, 522]. The bacterial system comprises an FAD- and NAD(P)H-binding FDR and an FD, with both components being cytoplasmic, and as typified by the well-studied PDR and the 2Fe–2S cluster-binding PD in the *Pseudomonas putida* P450cam system [523]. This type of system also appears in the eukaryotic mitochondrion (notably in the mammalian adrenal gland, and consistent with the endosymbiont theory of the evolution of this organelle), and is associated with driving catalysis of various P450s involved in steroid hormone biogenesis and degradation [524, 525]. Here, the P450s are anchored in the mitochondrial membrane, as is the NADH-dependent ADR, with the FD component being the cytoplasmic 3Fe–4S cluster-binding adrenodoxin [526]. The eukaryotic class II system exploits the NADPH-dependent, FAD- and flavin mononucleotide (FMN)-binding CPR, which is anchored in the endoplasmic reticulum by an N-terminal transmembrane domain [527]. The colocation with P450s enzymes again facilitates productive interactions. However, from the 1980s onwards the greater complexity and variability of microbial P450 redox systems was increasingly recognized, first through studies by Armand Fulco's group on the *Bacillus megaterium* P450 BM3 (CYP102A1) fatty acid hydroxylase (see the section 'Microbial P450-(redox) partner fusion enzymes' for more details on the catalytic mechanism of P450 BM3). The BM3 system results from fusion of a cytoplasmic P450 (N-terminus) to a soluble CPR (again devoid of an N-terminal membrane anchoring region) via a flexible peptide linker region, the length of which appears to be more important than its specific amino acid composition [528, 529]. It is thus an evolutionary adaptation of the eukaryotic class II system, and has a much higher catalytic rate than the eukaryotic class II enzymes [530]. As described in the following section, BM3 is predominantly dimeric in solution [531, 532], suggesting that the inter-domain linker length is optimized to facilitate efficient communication of the CPR FMN domain with both its electron donor (the CPR FAD cofactor) and its acceptor (the P450 domain heme iron). BM3 and related enzymes catalyze rapid oxidation of fatty acids near the ω-methyl group (typically hydroxylation at ω-1 to ω-3 positions), but despite being extensively studied to understand its molecular properties, BM3's physiological function remains obscure, although suggestions have been made—e.g., involvement in metabolism of toxic unsaturated fatty acids derived from plants [533].

The 'floodgate' of complexity of microbial P450 redox systems has steadily opened since the discovery of BM3 as the first example that violated the class I/class II paradigm. In recent years, the numbers of novel systems formed as genetic fusions of partial or complete redox partner systems with their cognate P450s has expanded considerably. A major factor underlying this expansion of novel fusion systems is the advent of high-throughput microbial genome sequencing, enabling the rapid identification of such gene fusions using bioinformatics tools, e.g., the CDART (Conserved Domain Architecture Retrieval Tool) program which searches for conserved domain organization when provided with the sequence of, e.g., a particular type of P450-redox partner fusion enzyme [534]. However, a consequence here is that many such recently annotated P450-partner fusion enzymes remain uncharacterized (see the section 'Microbial P450-(redox) partner fusion enzymes'). In contrast, identification of new types of P450 redox systems where novel types of nonfused electron carrier proteins are used has been achieved mainly through direct experimentation. Key examples are discussed below, with Fig. 6.29 illustrating key pathways of electron transfer to cytochromes P450.

6.3.1 Diverse FD Partners

The model system P450cam has its well-characterized PDR and PD partners encoded together on the CAM plasmid, along with other genes that enable the *Pseudomonas putida* host to grow on camphor as a sole carbon source [535]. The 2Fe–2S cluster in PD has a midpoint reduction potential (E_m) of −240 mV (vs. the normal hydrogen electrode, NHE) for the $[2Fe–2S]^{2+}$ to $[2Fe–2S]^{1+}$ redox couple. This value is nicely poised such that NADH-derived electrons (E_m=−320 mV) can be transferred from the PDR flavoprotein (E_m=−230 mV for the FAD/$FADH_2$ couple in NAD^+ -bound PDR) to reduce PD [536]. Thereafter, the reduction of P450cam itself is regulated by substrate binding, with the E_m for the P450 heme Fe^{3+}/Fe^{2+} couple being approximately −300 mV in the absence of substrate, and increasing to approximately −170 mV in the camphor-bound form [537]. Crystallographic studies and molecular modeling revealed that the PD/P450cam partner-binding interface occurs at the proximal face of the heme cofactor, and occurs across a relatively small interaction interface in which there is strong shape complementarity between the partners (Fig. 6.30a). A similar type of recognition process likely governs productive, transient interactions between other FDs and their microbial P450 partners; for instance, in the case of the *Pseudomonas* sp. 2Fe–2S ferredoxin terpredoxin, which is the electron donor to P450terp (CYP108A1) that functions in the oxidation of α-terpineol to facilitate its hydroxylation for energy extraction by the bacterium [120]. A 2Fe–2S ferredoxin (Fd_{bisD}) from *Sphingomonas* sp. strain AO1 was also shown to support the activity of the $P450_{bisD}$ in a pathway for oxidative degradation of bisphenol A, whereas a heterologous spinach FDR/FD system supported only very weak activity [538]. However, various studies have also reported that microbial P450 activity can be driven by the heterologous spinach FDR and 2Fe–2S FD, including work by Makino et al., who showed that the *Streptomyces griseus* CYP154C3 catalyzes monooxygenation of a range of steroids using this system [539]. However, other types of FD were also reported to support the activities of bacterial and archaeal P450 enzymes. For instance, 3Fe–4S FD are located chromosomally adjacent to two P450 enzymes (CYP51B1 and CYP143) in *M. tuberculosis* [540]. The Fer1 protein (product of gene *Rv0763c,* adjacent to *CYP51B1*) protein was expressed and purified from *E. coli,* and shown to support electron transfer to CYP51B1, and the P450-dependent oxidation of dihydrolanosterol [21]. In unpublished work, the *Rv1786* gene product (Fer2) was also expressed and shown to bind a 3Fe–4S cluster by EPR studies (McLean KJ et al., unpublished work), and should thus be the preferred partner of the uncharacterized *M. tuberculosis* CYP143 P450. In addition, Guengerich's group characterized redox partner specificity for the *S. griseus* CYP105D5 in fatty acid hydroxylation, investigating interactions with each of the six 3Fe–4S FD from the bacterium, and establishing that best activity was

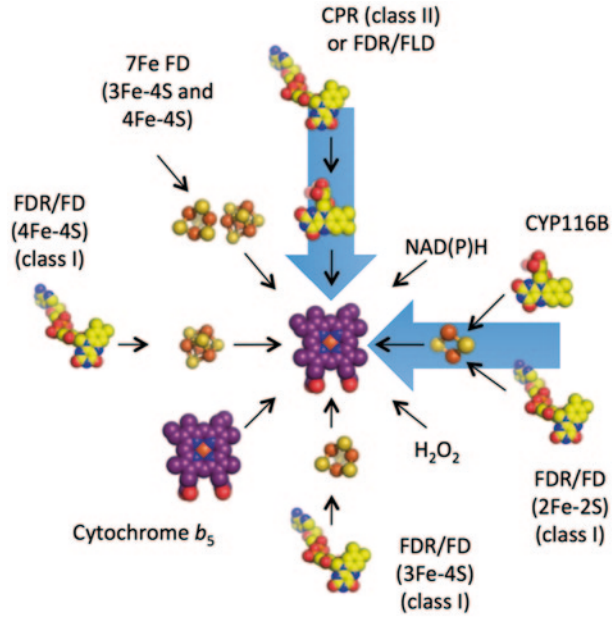

Fig. 6.29 Diagram of P450 electron transfer pathways and cofactors. The schematic shows the extent of current knowledge on major pathways of heme reduction in P450 enzymes. The main pathways are indicated by *thick blue arrows*. Going clockwise from the top: (i) The class II redox system with electrons donated by NADPH and passed through FAD and then FMN cofactors in CPR to the P450. Alternatively, electron transfer through an FAD-binding flavodoxin reductase and a flavodoxin, as seen for P450cin with cindoxin [559]. (ii) Direct reduction of P450 heme iron by NAD(P)H, as seen in the CYP55A1 nitric oxide reductase from *Fusarium oxysporum* [610]. (iii) Heme iron reduction from a 2Fe–2S ferredoxin, either using a separate NAD(P)H-dependent ferredoxin reductase (the class I P450 redox system) or from NAD(P)H via an FMN cofactor contained within the same phthalate dioxygenase reductase-like (PDOR) protein as the ferredoxin in the CYP116B family P450-PDOR fusion proteins [141]. (iv) Direct conversion of the ferric heme iron to the compound 0 (ferric–hydroperoxo) form, as seen naturally in P450 peroxygenases such as the fatty acid decarboxylase OleT [575]. (v) Class I-type system using a 3Fe–4S ferredoxin, as seen in the case of the *M. tuberculosis* CYP51B1 and its ferredoxin partner (Rv0764c, Fer) [21]. (vi) P450 reduction by cytochrome b_5 in eukaryotic P450 systems. Due to its positive potential, it is likely that b_5 delivers the second electron required for oxidative catalysis, with electrons derived from NADH via an FAD-binding b_5 reductase [505]. (vii) class I-type system using a 4Fe–4S ferredoxin, as is observed for fatty acid hydroxylation by *B. subtilis* P450 BioI when driven by an NAD(P)H-dependent FAD-binding reductase [543]. (viii) non-NAD(P)H-dependent archaeal redox partner system using a pyruvic acid and CoA-dependent reductase system and a 7Fe (4Fe–4S and 3Fe–4S cluster containing) ferredoxin [95]. NADPH nicotinamide adenine dinucleotide phosphate, FAD flavin adenine dinucleotide, FMN flavin mononucleotide

achieved with the FDR1 FDR and the ferredoxin Fdx4 [541]. These data suggest that, in microbes with multiple FD genes, there is likely to be selectivity for preferred P450 partners, and that interactions between FDR and FD proteins may also vary in efficiency. In the P450 BioI enzyme (CYP107H1) from *Bacillus subtilis,* early studies demonstrated that fatty acid hydroxylation by the P450 can be driven by an exogenous (*E. coli*) or host FDR together with a 4Fe–4S FD from the host bacterium (Fer). However, other types of redox partners are also able to support fatty acid hydroxylation by BioI [542, 543], while interactions with other another (nonredox) protein results in a different catalytic outcome involving lipid C–C bond cleavage (see below for further information). Thus, each of the 'common' forms of FD was shown to support catalytic functions of P450s from the same organisms, although surrogate FDR/FD partners can also support bacterial P450s, often with comparable (or higher) efficiency in vitro, for instance, in the oxidation of 4-cholesten-3-one by the *Mycobacterium smegmatis* CYP125A3 and CYP142A2 P450s [367].

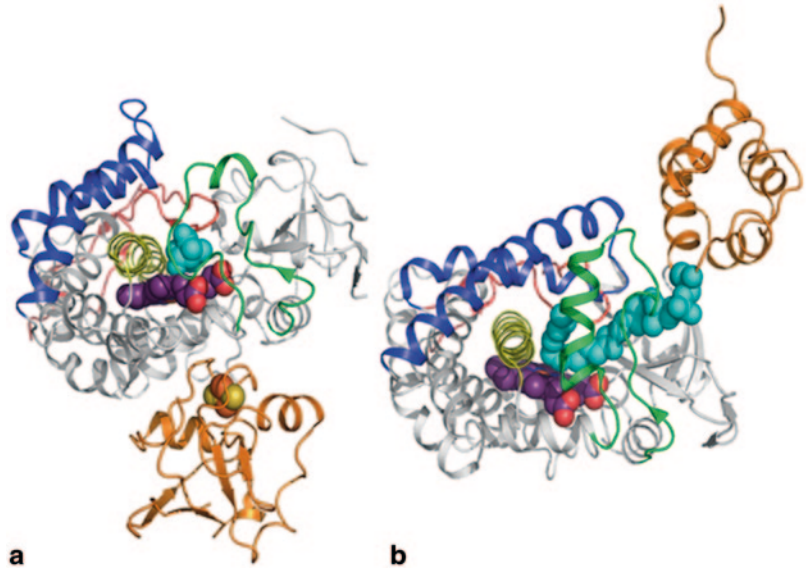

Fig. 6.30 P450 protein/partner complexes. A cartoon view is shown for two crystal structures documenting distinct P450 protein/partner protein interactions. Panel A depicts the P450cam (CYP101A1)–putidaredoxin (PD) complex with the redox partner depicted in *orange* (PDB 4JWS). The 2Fe–2S cluster is in close vicinity of the heme, with the PD docking on the proximal face of the P450 near to the fifth ligand cysteinate–iron bond region [770]. In contrast, panel B shows the interaction observed between P450 BioI (CYP107H1) and its acyl carrier protein (ACP) partner linked to the lipid substrate (PDB 3EJB, 3EJD, and 3EJE) [131]. In this case, the partner protein binds at the opposite side of the P450, near the BC loop and FG helices. This suggests that, in the case of BioI, a ternary complex between BioI–ACP and the electron transfer partner remains possible, and is likely essential to facilitate the oxidative cleavage of the ACP-bound lipid substrate

Recently, an unusual type of 3Fe–4S cluster was identified in an FD (HaPuxC) from the Gram-negative purple nonsulfur bacterium *Rhodopseudomonas palustris* HAa2. HaPuxC has a histidine residue in a position in its iron–sulfur cluster-binding motif that is normally occupied by the fourth iron-coordinating cysteine in 4Fe–4S iron–sulfur clusters, or by an alanine or a glycine in typical 3Fe–4S ferredoxins [544]. A similar histidine-containing motif was observed in other 3Fe–4S ferredoxins, including ones from mycobacterial strains [21, 545]. HaPuxC binds a 3Fe–4S cluster and the protein has a fold typical of 3Fe–4S and 4Fe–4S ferredoxins, but its crystal structure reveals some differences (e.g., length of *beta* sheet elements) compared to other FD. The histidine is positioned close to where a fourth iron atom would be found in a 4Fe–4S cluster, but its side chain is oriented away for the cluster and the imidazole group nitrogen atoms make hydrogen-bonding interactions with glutamate carboxylate oxygens on an adjacent loop, and with S2 of the iron–sulfur cluster. Interestingly, this type of FD is encoded adjacent to *CYP194A* subfamily P450 genes in another *R. palustris* strain and in *Bradyrhizobium japonicum* USDA110, indicating specificity of this type of FD for these CYP194A P450s [544]. An even more unusual type of FD (a Zn- and 7Fe-containing FD) was also reported to be part of a non-NAD(P)H-dependent redox system driving the *Sulfolobus solfataricus* CYP119A2 enzyme, as discussed further in the section ‘P450s from thermophilic microbes and novel redox systems for Sulfolobus P450s’ [95].

6.3.2 Flavodoxins as Bacterial P450 Redox Partners

Given that the general structure of the FMN-binding domain of eukaryotic CPR enzymes is highly related to that of microbial flavodoxins, it is perhaps not surprising that bacterial flavodoxins have been shown to act as redox

partners for P450s from both homologous and heterologous organisms [115, 546]. Jenkins and Waterman reported that the bovine steroid 17α-hydroxylase/17,20-lyase P450 enzyme (P450c17, CYP17A1) heterologously expressed in *E. coli* was functional in this bacterium, and went on to identify the enzyme system responsible as the NADPH-dependent flavodoxin (ferredoxin) reductase (FLDR) and flavodoxin (FLD). Further evidence for the binding of flavodoxin to P450c17 came from a type I spectral shift (low spin towards high spin) in the P450 ferric heme iron induced upon titration with the *E. coli* FLD (K_d ~0.2 μM) [547]. The productive interaction of the FLD was shown to be more sensitive to elevations in ionic strength of the medium compared to that of the rat CPR enzyme, and based on the accumulation of the blue semiquinone (SQ) form of the *E. coli* FLD in in vitro assays with P450c17, it was concluded that the FLD SQ was the relevant electron donor to the P450 [548]. However, based on thermodynamic grounds, reduction of the P450 heme iron by the *E. coli* FLD hydroquinone (HQ) (E_m = −433 mV for the SQ/HQ couple, compared to −254 mV for the OX/SQ couple) may be more likely, at least for the first electron transfer to the heme iron [549]. *E. coli* FLDR/FLD were also shown to support successive oxidations of pentalenene to pentalen-13-al via pentalen-13-ol in an NADPH-dependent manner, catalyzed by the *Streptomyces avermitilis* CYP183A1 in the bacterial pathway to biosynthesis of the sesquiterpene lactone antibiotic pentalenolactone [550]. Other P450 systems shown to be supported by *E. coli* FLDR/FLD include CYP152A1 from *Clostridium acetobutylicum* (which catalyzes α-hydroxylation of fatty acids), although this P450 can be driven more effectively by the P450 BM3 reductase domain, as well as by hydrogen peroxide [551]. *E. coli* FLDR/FLD also support the catalysis of *S. coelicolor* A3 (2) CYP170A1 in successive oxidations of the tricyclic hydrocarbon *epi*-isozizaene, producing first an epimeric mixture of albaflavenols, and then the sesquiterpene antibiotic albaflavenone [202].

The *B. subtilis* BioI P450 protein (CYP107H1) was identified as a novel gene in the bacterium's biotin gene cluster, and implicated in the synthesis of an intermediate at or before the formation of the C7 dicarboxylic acid pimelate [552]. BioI was first reported as a fatty acid binding and hydroxylating P450 involved in biotin production in this bacterium, and a heterologous redox partner system was shown to support BioI-mediated hydroxylation of long-chain fatty acids [553, 554]. However, the relationship of these reaction products to a biotin synthesis pathway was unclear. Instead, De Voss and coworkers presented a model in which *E. coli* ACP that co-purifies with BioI presents the P450 with a fatty acid substrate that is covalently linked to the ACP (Fig. 6.30b). Provision of a heterologous redox partner system enables consecutive oxidations of adjacent mid-chain (C7 and C8) C–H bonds in the substrate, leading to C–C bond cleavage and the production of pimelic acid to be used as a precursor in biotin synthesis [64, 555] (Fig. 6.31). Structural data for lipid-loaded ACP–P450 complexes (using three different lipid chain lengths) revealed binding modes for the substrates that were consistent with this observed bond cleavage activity [131]. While this research points clearly to the mechanism by which BioI should participate in biotin synthesis, the preferred redox partners for the oxidative cleavage reaction are uncertain, with both FD (the *B. subtilis* Fer protein) and flavodoxins being the potential ultimate electron donors to the BioI P450. *B. subtilis* encodes two short-chain flavodoxins (YkuN and YkuP) and UV-visible and fluorimetric titrations with the BioI protein indicated that both FLDs bind to BioI, while stopped-flow absorption reactions between reduced YkuN/YkuP and BioI revealed heme iron reduction rate constants of ~2.5 s^{-1}, considerably faster than that achieved using *E. coli* FLD. Reconstitution of BioI with NADPH/*E. coli* FLDR and either of the YkuN/YkuP FLDs resulted in fatty acid hydroxylation. These data reinforce the fact that the BioI P450 is functional in lipid hydroxylation with both FLD and FD partners, but requires the fatty acid-loaded ACP partner to achieve in-chain lipid bond cleavage reactions required to produce the biotin pathway intermediate [556]. The role of bacterial flavodoxins in BioI-dependent fatty acid hydroxylation thus remains uncertain here, although

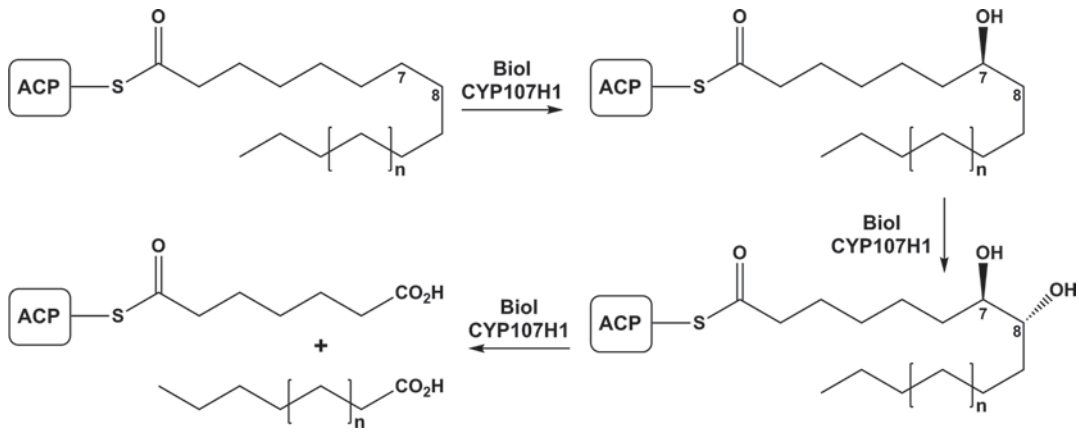

Fig. 6.31 The C–C bond cleavage reaction catalyzed by P450 BioI. The reaction schemes show the three-step oxidative reaction that leads to bond cleavage between the C7 and C8 carbon atoms of acyl carrier protein (ACP)-linked fatty acids by BioI (CYP107H1) from *B. subtilis* in the formation of pimelic acid, an intermediate in the bacterial biotin synthesis pathway [131]

the high affinity of BioI for fatty acids (e.g., K_d values of 0.4 and 5.2 μM for palmitoleic acid and pentadecanoic acid, respectively) suggests that a proportion of the enzyme would be fatty acid bound in the cell, and thus prone to undergoing fatty acid oxidation reactions through an FDR and FD/FLD-driven electron transfer process [557]. *B. subtilis* FDR protein(s) and YkuN/YkuP/Fer are also clearly candidates for electron transfer to the ACP–BioI complex to facilitate the consecutive P450 oxidation reactions needed for cleavage of the ACP-bound lipid substrate in the bacterium.

The first definitive proof of the involvement of a flavodoxin as an electron donor to a microbial P450 came from studies of the cineole-oxidizing P450cin (CYP176A1) from *Citrobacter braakii,* a P450 catalyzing the monooxygenation of the terpene 1,8-cineole [121]. Here, the *C. braakii* flavodoxin cindoxin (Cdx) and *E. coli* FLDR (surrogating for the native cindoxin reductase) were shown to be an effective redox partner system for P450cin, with a reported k_{cat} of ~300 min^{-1} for cineole-dependent NADPH oxidation [558]. The standard redox potentials for the oxidized/SQ (E_1) and SQ/HQ (E_2) redox couples of the Cdx FMN were established by spectroelectrochemical methods as $E_1 = -93$ mV and $E_2 = -226$ mV, consistent with the Cdx HQ being the relevant electron donor to the ferric, cineole-bound P450cin ($E_m = -202$ mV) [559]. Crystal structure determination of P450cin and Cdx enabled modeling that predicted Arg346 (in P450cin) to form a salt bridge with Asp94 (in Cdx), which in turn makes an electrostatic/polar interaction with the Cdx FMN ribityl hydroxyl group. Tyr96 (adjacent to the FMN isoalloxazine shielding/stacking aromatic residue Tyr97) is also predicted to protrude into a hydrophobic cavity in P450cin, and to interact with P450cin Arg102. The P450cin R102A and R346A mutations resulted in an approximately tenfold decrease in the rate constant for cineole-dependent NADPH oxidation in an FLDR/Cdx/P450cin system, while a Cdx Y96L mutant had a similar effect, and also resulted in approximately threefold decreases in the rate constants for Cdx-dependent P450cin reduction and FLDR-dependent Cdx reduction [558]. Thus, diminished catalytic efficiency in these mutants is consistent with their potential involvement in the docking interface between P450cin/Cdx, and possibly in the electron transfer mechanism. Other examples of microbial P450s in which catalytic activity can be supported by an FLD protein include CYP106A1 from *Bacillus megaterium* DSM319, where both the host FLD protein and three of four host FD (containing either 3Fe–4S or 4Fe–4S clusters)

supported CYP106A1-mediated oxidation of the anti-inflammatory pentacyclic triterpene 11-keto-β-boswellic acid (KBA) to 7β-hydroxy-KBA and other side products in the presence of NADPH and the *Schizosaccharomyces pombe* Arh1 flavoprotein reductase enzyme [133]. The *Clostridium acetobutylicum* fatty acid hydroxylase CYP152A2 (known as a peroxygenase P450—see the section 'P450 systems that bypass redox partners') was also catalytically active when reconstituted with one of the two FLDs from the host organism (CacFld1), *E. coli* FLDR and NADPH, with the second FLD (CaFld2) not reduced effectively by FLDR/NADPH. Both α- and β-hydroxy myristic acid products were formed [560].

While FD are 'pure' one-electron carriers in their P450-reducing role, the situation is clearly more complex with the flavodoxins. The FLD FMN cofactor can occupy the SQ (one-electron reduced form) as well as the HQ (two-electron reduced form), with either form being a potential electron donor. In reality, the vast majority of microbial flavodoxins are found to have widely differing E_m values for their first electron (OX/SQ) and second electron (SQ/HQ) reduction couples. The OX/SQ couple is generally quite positive (e.g., −254 and −149 mV vs. NHE for the *E. coli* and *Desulfovibrio vulgaris* FLDs, respectively), while the SQ/HQ couple is much more negative (e.g., −433 and −438 mV vs. NHE, respectively) [549, 561]. This typically results in the intracellular state of bacterial FLDs being predominantly SQ, and meaning that the relevant redox couple for P450 reduction is the SQ/HQ transition, which is also the case for eukaryotic P450 reduction by CPR. An interesting outlier to this generalization is the P450 BM3 enzyme, where electron transfer to the P450 heme comes from the FMN anionic SQ [562, 563] (see the section 'Microbial P450-(redox) partner fusion enzymes').

6.3.3 P450s from Thermophilic Microbes and Novel Redox Systems for Sulfolobus P450s

The first report of the isolation of a thermostable P450 was for a progesterone 6β-hydroxylase from the moderately thermophilic bacterium *Bacillus thermoglucosidasius* strain 12060. The enzyme was purified to near homogeneity from the host organism and was shown to form characteristic Fe^{2+}–CO complex at 449 nm, with ~60 % activity retained at 70 °C. However, a host redox partner system was not identified [104, 105]. The X-ray crystal structure for CYP231A2 from the thermoacidophile *Picrophilus torridus* was solved, indicating that compact structural organization of this small P450, along with short loop structures, were important determinants of its thermostability (rather than clustering of amino acid residues or salt-bridge networks seen in other thermostable P450s). However, a redox partner system was not identified for CYP231A2 [103]. CYP154H1 from the moderately thermophilic actinobacterium *Thermobifida fusca* was expressed and purified in *E. coli,* and was shown to have a melting temperature (T_m) of ~67 °C. Catalytic activity was reconstituted using the *P. putida* PDR and PD class I redox system, demonstrating that a range of small aromatic molecules could be oxidized (e.g., epoxidation and hydroxylation of styrene, and transformation of a series of arylaliphatic sulfides to their corresponding sulfoxides), with a rate constant determined for product formation of 0.31 min^{-1} with styrene as substrate, considerably slower than the P450cam enzyme [194]. However, a host redox partner system was identified for the thermostable CYP175A1 from the Gram-negative eubacterium *Thermus thermophilus*. CYP175A1 was expressed and purified from *E. coli* and shown to have a T_m of 88 °C. Crystal structure data indicated that networks of salt bridges, in addition to shortening of loops and interconnecting regions (by comparison with mesophilic P450s), were among the major determinants of CYP175A1 thermostability [101]. A redox partner system supporting CYP175A1 catalytic activity was isolated from *T. thermophilus* cell extract by fractionation of proteins able to support β-carotene hydroxylation to β-cryptoxanthin. This approach identified two partially purified CYP175A1 redox partner proteins with UV-visible spectral features indicative of the presence of flavin and iron–sulfur cofactors, respectively. The proteins were then puri-

fied to homogeneity and identified in the genome sequence as a 7Fe ferredoxin (i.e., a FD binding both 3Fe–4S and 4Fe–4S iron–sulfur clusters) using N-terminal amino acid sequencing, and as a ferredoxin $NAD(P)^+$ reductase (mis-annotated in the *T. thermophilus* genome as a thioredoxin reductase (TR)) using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. This FDR protein was shown to bind FAD noncovalently and to show marked preference for NADPH over NADH (K_m values of 4.1 μM and 2.4 mM, respectively, in ferricyanide reduction assays) [564]. The *T. thermophilus* FDR and 7Fe FD have T_m values of >110 and 99 °C, respectively, based on retention of activity in ferricyanide reduction assays subsequent to 30-min incubations at temperatures between 40 and 110 °C. In addition, the *T. thermophilus* FDR partner was proposed to be a novel type of FDR enzyme, due to its high sequence similarity with TR enzymes, but the lack of TR activity through the absence of a redox active site (CXXC) motif. The *T. thermophilus* FDR is closely related to FDR proteins from *B. subtilis* (YumC) and *Chlorobium tepidum,* with phylogenetic analysis suggesting that these types of enzymes may be a new subclass of FDRs within the glutathione reductase class of FDR enzymes [564–566]. Catalytically active fusion enzymes were also produced by linking *CYP175A1* to the *T. thermophilus* FDR and FD genes (with short peptide linkers between the individual components) to make constructs expressing H_2N–CYP175A1–FDR–FD–COOH (175RF) and H_2N–CYP175A1–FD–FDR–COOH (175FR) proteins. The V_{max} value for the 175RF protein (17.9 min^{-1}) was much higher than that of 175FR (0.7 min^{-1}) or of an equimolar mixture of the individual proteins (1.9 min^{-1}) in β-carotene hydroxylation assays, demonstrating the production of a thermostable, catalytically self-sufficient fusion enzyme [567].

The P450 systems characterized from species of the acidophilic and thermophilic *Sulfolobus* genus have provided important members of the P450 superfamily from which crucial data on the nature of compound I have been obtained, as well as interesting insights into novel systems driving P450 catalysis. Studies on the P450s CYP119A1 and CYP119A2 from the archaeons *Sulfolobus acidocaldarius* (previously assigned as *Sulfolobus solfataricus*) and *S. tokodaii* strain 7 (also known as P450st) also provided important insights into the mechanisms by which these P450s gain thermostability [568]. An extended network of aromatic amino acids around CYP119A1 was observed in the crystal structure of the enzyme, and proposed to be the major determinant of thermostability in this P450, which has a T_m = 91 °C [91, 92] (Fig. 6.3). This hypothesis was supported by mutational analysis which demonstrated that substituting various aromatic amino acids in the cluster for alanines resulted in decreases in T_m of up to 15 °C, while mutations designed to disrupt a salt bridge between residues in the F/G loop (Arg154) and the I-helix (Glu212) affected activity, but not P450 thermostability [569]. In the case of CYP119A1, this P450 was shown to bind lauric acid with a typical type I (low- to high-spin ferric) heme iron shift, and with a K_d value of 1.1 μM. Moreover, CYP119A1 catalyzed the regio-selective (ω-1) hydroxylation of lauric acid (with a small proportion of ω-hydroxylauric acid product) with a k_{cat} of 10.8 min^{-1}, with electrons from NADH provided by the *P. putida* PDR and PD redox partner system [99]. However, the introduction of the mutations T214V (to improve lauric acid binding and substrate-induced heme iron spin-state change) and D77R (to enhance binding of PD) enabled a 15-fold increase in fatty acid hydroxylase activity relative to WT CYP119A1 with PDR/PD partners [98].

However, a more unusual CYP119A1 redox partner system was identified by Ortiz de Montellano's group, following studies by Fukuda et al. to identify an archaeal 2-oxoacid:ferredoxin oxidoreductase (OFOR) enzyme system that catalyzes the coA-dependent decarboxylation of 2-oxoacids (pyruvate and 2-oxoglutarate) using a Zn–7Fe ferredoxin as an electron acceptor [570]. Puchkaev et al. reported the activation of *S. acidocaldarius* CYP119A1 in lauric acid hydroxylation using the α/β subunit-containing OFOR and the FD from the closely related *S. tokodaii* strain 7, when reconstituted with coA and pyruvic acid. Acetyl CoA and CO_2 are the other products of the reaction, and the fatty acid hydroxylase ac-

tivity of the system increased consistently as the reaction temperature was elevated from 20° up to 70 °C. While catalytic rates were not particularly high (~0.25 min^{-1} at 70 °C), these results provided the first evidence supporting the ability of non-NAD(P)H-dependent redox proteins to drive P450 catalysis [95, 96].

Studies on the CYP119A2 enzyme showed that this P450 could catalyze styrene epoxidation in absence of redox partner enzymes, and with only the addition of either NADH or NADPH to the P450. Structural studies showed that the heme-binding pocket of CYP119A2 is large enough to accommodate NAD(P)H [93, 97]. Studies were done using both the WT CYP119A2 and a mutant in which the F/G loop region was deleted (ΔLeu151-Glu156). WT CYP119A2-catalyzed NADH-dependent styrene epoxidation slightly better than with NADPH, but only ~6% of the NADH oxidized was coupled to styrene epoxidation, with an NADH K_m of 13 mM. The catalytic rate was similar for the CYP119A2 deletion mutant, but the NADH K_m was improved somewhat to 7 mM. The apparent affinity for NADH was much weaker than that for styrene (0.29 and 0.59 mM for WT CYP119A2 and the deletion mutant, respectively). The open nature of the CYP119A2 heme-binding cavity was considered comparable to that found in the NAD(P)H-dependent nitric oxide reductase P450nor (CYP55A1) (see the section 'P450 systems that bypass redox partners'). Binding of NAD(P)H close to the heme in CYP119A2 was proposed to be stabilized by arginine residues near the entry to the active site, and a distinct channel identified was proposed to be involved in water and/or proton relay to the active site. The improved K_m for NADH in the deletion mutant is likely explained by improved cofactor access to the active site on removal of the F/G loop region [97]. CYP119A2 can also be driven by the peroxide shunt method using H_2O_2, with a k_{cat} value of 9.5 min^{-1} for ethylbenzene hydroxylation at pH 10, albeit with a high K_m value of 26.2 mM for H_2O_2 [571]. CYP119A1 was also shown to catalyze H_2O_2-dependent styrene epoxidation with a k_{cat} of 78.3 min^{-1} and a K_m of 9.2 mM for H_2O_2 [568]. However, the most important use of the peroxide shunt in the characterization of a thermostable P450 was undoubtedly achieved by Rittle and Green through reacting *meta*-chloroperbenzoic acid (m-CPBA) with CYP119A1 to enable formation of the reactive compound I intermediate and its definitive identification and characterization [13].

Potentially, *Sulfolobus* spp. and other thermostable P450s have important industrial/biotechnological applications in view of their high stability. However, this goal will likely only be achieved through identification of thermophile P450s with relevant activities, or by protein engineering to introduce desired functions, or possibly by learning lessons from thermophilic P450s to engineer thermostability into mesophilic P450 that already possess useful activities. These approaches will likely be challenging in view of the potential effects on protein stability in the former case, and through introduction of greater rigidity in the latter case—which could be deleterious to conformational dynamics required for P450–substrate interactions and catalysis.

6.3.4 P450 Systems That Bypass Redox Partners

In microbes, there are excellent examples of P450 systems that have evolved activities that are independent of electron input from protein partners. The ability to drive P450 oxidations using hydrogen peroxide (H_2O_2) or organic peroxides (such as m-CPBA and iodosylbenzene) is well known [102, 572]. This is rationalized through the ability of such molecules to interact with the P450 ferric heme iron and convert it directly to compound 0 (ferric–hydroperoxo), which thereafter undergoes a further protonation and a dehydration to generate the reactive compound I [573] (Fig. 6.4). However, the nonspecific reactivity of H_2O_2 with both the protein and the heme cofactor generally means that this is not a highly effective means of driving P450 catalysis, since enzyme inactivation competes with a productive catalytic process. Nevertheless, nature has clearly adopted this mechanism and has produced 'peroxygenase' P450s that have evolved to perform this reaction efficiently. The properties of the best-char-

acterized examples of such P450s are discussed below.

6.3.4.1 The Alkene-Producing OleT P450

In the marine alkaliphilic bacterium *Jeotgalicoccus* sp. ATCC 8456, the OleT P450 (CYP152L1) catalyzes H_2O_2-dependent decarboxylation of long-chain fatty acids to produce terminal alkenes, and is viewed as an enzyme with potential in fine chemical/biofuel production [574, 575]. Rude et al. detected linear and branched terminal alkenes (C18–C21) in a number of *Jeotgalicoccus* strains, indicating that ability to decarboxylate fatty acids to produce terminal alkenes was common to bacteria of this genus. Fractionation of the decarboxylase activity from bacterial cell extracts enabled its identification as a P450 and for the subsequent cloning and expression of the *CYP152L1* gene in *E. coli*. The expression cells were shown to produce 1-pentadecene, reflecting the abundance of hexadecanoic acid (palmitic acid) substrate in the bacterium. The diunsaturated 1,10-heptadecadiene was also detected, as was 1-heptadecene following addition of exogenous octadecanoic acid (stearic acid) to the OleT expression strain [557, 574, 576]. In vitro studies demonstrated that the fatty acid decarboxylation by OleT was driven effectively by H_2O_2, and thus did not require a redox partner system, a result consistent with the structural relationship of this P450 with other enzymes of the CYP152 family that were shown previously to catalyze H_2O_2-dependent fatty acid hydroxylation—most notably the well-studied *B. subtilis* CYP152A1 (P450 BS_β) and *Sphingomonas paucimobilis* CYP152B1 (P450 SP_α) P450s [577, 578]. The CYP152A1/B1 enzymes are discussed in more detail below, and collectively these H_2O_2-dependent types of P450s are termed peroxygenases [579]. Interestingly, addition of dithiothreitol (DTT) was shown to facilitate production of n-1 alkenes from tetradecanoic (myristic), palmitic and the C20 eicosanoic (arachidic) acid, possibly through production of H_2O_2 by reaction of DTT with oxygen in the presence of the heme iron [574, 580]. However, DTT was also show to be a reasonably effective inhibitor of OleT (K_d = 159 μM), binding to the heme iron and inducing a hyperporphyrin (split Soret) spectrum indicative of coordination in the distal position by DTT in both its thiol and thiolate forms [575]. Comparative studies of the products of P450s SP_α and BS_β from turnover of palmitic acid with H_2O_2 indicated that while BS_β generated 1-pentadecene as a minor product compared to α- and β-hydroxy palmitic acid, only the α-hydroxy palmitic acid was formed by SP_α, with no alkene detected. OleT produced small amounts of the α- and β-hydroxy acids, but functions mainly as a decarboxylase with an ~17.5-fold greater ratio of 1-alkene to (combined) hydroxylated fatty acids formed in comparison to BS_β [574].

The OleT P450 was found to aggregate in low-salt buffer conditions, likely consistent with the halophilic nature of its host bacterium. Unusually for a P450, resolubilization of the OleT pellet in high-salt buffer resulted in a fully active, heme-bound P450. The purified, low-spin OleT has a Soret maximum at 418 nm, and forms a CO-bound complex with A_{max} at 449 nm, consistent with retention of a cysteine thiolate ligand on reduction and CO binding [575]. The binding of several fatty acids produced low- to high-spin shifts in the OleT heme iron spin-state equilibrium, consistent with their binding in the environment of the heme and causing displacement of a water ligand in the sixth position on the heme iron. Tight binding of various long-chain fatty acids was established, with a K_d of 0.29 μM determined for arachidic acid. The reduction potential for the ferric/ferrous transition of the OleT heme iron was determined by spectroelectrochemical methods in both substrate-free and arachidic acid-bound forms, but indicated negligible change in E_m, despite the extensive development of high-spin heme iron in the fatty acid-bound form (E_m values vs. NHE of −103 ± 6 mV for substrate-free and −105 ± 6 mV for arachidic acid-bound OleT). The potentials are quite positive compared to those for other microbial P450s (e.g., −368 mV for substrate-free P450 BM3 heme domain, compared to −239 mV for the arachidonic acid-bound form) [575, 581]. The data for OleT indicate that perturbation to heme redox potential by substrate is not crucial for enzyme activation in this enzyme, and that the proximity

of the negatively charged fatty acid carboxylate group may offset any positive shift in heme iron potential induced by displacement of the sixth water ligand and accompany development of high-spin heme iron. Transient kinetic studies on OleT were done using stopped-flow absorption spectroscopy, and by mixing H_2O_2 (at various final concentrations) with arachidic acid-bound OleT. The kinetics of conversion of the substrate-bound (high-spin) OleT heme iron to the low-spin form were monitored at 417 nm, in this way following the process of H_2O_2-induced activation of the heme and decarboxylation of the fatty acid substrate. A second-order dependence of reaction rate on peroxide concentration was observed (0.80 ± 0.02 s^{-1} μM^{-1} H_2O_2), with a turnover rate constant of 167 s^{-1} measured at the highest H_2O_2 concentration used (200 μM). An OleT K_d value for H_2O_2 of 10.4 μM was also estimated from these transient kinetic data [575]. The structure of OleT was determined by protein crystallography (using molecular replacement with the closely related BS_β P450), both in the substrate-free and the arachidic acid (C20:0)-bound forms (Fig. 6.32a) [575]. The two structures are highly similar to one another, and to P450 BS_β (Fig. 6.32b). Notable features in the OleT active site include a conserved arginine (Arg245), which makes the only direct, polar contact with the fatty acid carboxylate, near perpendicular to the distal face of the heme (Fig. 6.32c). Arg245 and the adjacent Pro246 are conserved in the I-helix of OleT, BS_β and SP_α, and replace the 'acid/alcohol' pair found in most class I and class II P450 enzymes (e.g., Asp251/Thr252 in P450cam and Glu267/Thr268 in P450 BM3) and which is associated with oxygen binding and activation [30, 33]. The evolutionary adaptation to enable a key protein-to-fatty acid carboxylate interaction in OleT and its relatives thus comes at the expense of disruption of the oxygen protonation machinery found in the vast majority of P450 oxygenases, but is again consistent with their divergence into a H_2O_2-dependent catalytic mechanism [575]. A histidine (His85) is located close to the substrate carboxylate, with a water molecular located between the two moieties. The His85 imidazole is directed towards the heme iron (at a distance of 5.8 Å) and sandwiched between Phe79 and the heme edge. The interstitial water

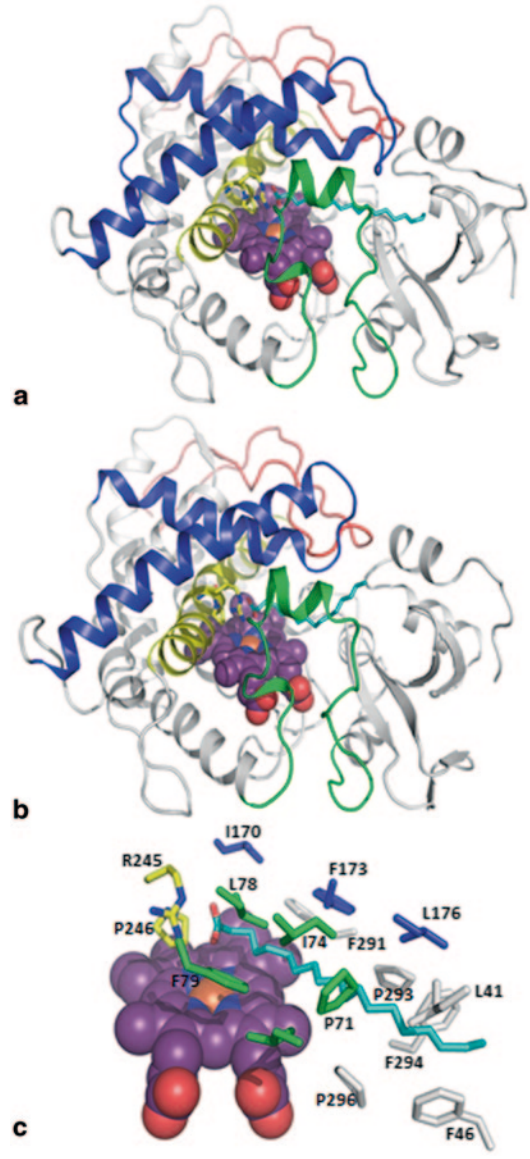

Fig. 6.32 Structures of P450 peroxygenases. A comparison between the fatty acid complexes of P450 OleT (panel **a**) (PDB 4L40) and the related BS_β (panel **b**) (PDB 1IZO). Both enzymes bind the substrate in similar manner, and key interactions occur between a conserved active site arginine residue (Arg245) and the fatty acid carboxylate moiety, as shown for OleT in panel C. P450 BS_β catalyzes predominantly fatty acid β-hydroxylation, while the OleT enzyme catalyzes oxidative decarboxylation of its long-chain fatty acid substrates to produce the n − 1 terminal alkenes [574, 575, 577]

does not interact with the heme iron as a sixth ligand (5.8 Å distant), and the iron is clearly pentacoordinate in the substrate-bound form. However, in the substrate-free OleT structure there are several poorly defined water molecules seen above the heme plane, an observation consistent with the more complex EPR spectrum derived for the substrate-fee OleT [575]. The OleT His85 is replaced by a glutamine in both the BS_β and SP_α P450s, suggesting a key role in regulating partition between hydroxylase and decarboxylase activities, possibly as a proton donating residue to a reactive iron–oxo intermediate in OleT [575]. The Q85H mutant of P450 BS_β was shown to result in a ~50% increase in catalytic rate of palmitic acid decarboxylation to 1-pentadecene. However, the major effects observed were a considerable increase in rate of palmitate β-hydroxylation together with a substantial drop in α-hydroxylase activity [574]. Recent studies showed that OleT could also catalyze NADPH-dependent fatty acid conversion to terminal alkenes in vitro when the P450 was fused to the PDOR domain of CYP116B2, or when provided with exogenous *E. coli* FLDR and FLD proteins. Unusually, the fatty acid preference was shifted to shorter chain lengths in the fusion protein compared to the H_2O_2-supported decarboxylase activity, possibly indicating an influence of the PDOR domain on active site structure. Production of terminal alkenes in *E. coli* strains expressing OleT was also shown [582]. This finding raises interesting questions as to the source of protons for catalysis if OleT can operate in vivo using a bacterial class I (or other) type of redox partner system. The absence of the acid/alcohol residue pair in OleT means that an uncharacterized proton relay system must support iron–oxo protonation reactions in such a case.

6.3.4.2 P450 BS_β, P450 SP_α and other bacterial peroxygenases

The *B. subtilis* BS_β (CYP152A1) and *S. paucimobilis* SP_α (CYP152B1) P450s were the first two microbial P450s characterized as peroxygenases, and both have been structurally characterized (Fig. 6.32b) [577, 578]. As noted above, these enzymes generate predominantly hydroxylated fatty acids, with SP_α giving exclusively the α-hydroxylated fatty acids in reactions with a range of fatty acids of C10 and above, with myristic acid (C14), pentadecanoic acid and arachidonic acid among the best substrates in terms of binding affinity and hydroxylation rate. Alkanes, fatty alcohols and aldehydes were not useful substrates, and the *S*-enantiomer products of fatty acids were obtained at >98% [583]. In contrast, BS_β produces α- and β-hydroxylated fatty acids from a similar range of fatty acids in an approximately 40:60 ratio, with both enzymes having a steady-state turnover number of ~1000 min^{-1} with their best substrates [577, 583–585]. Rude et al. confirmed that the SP_α P450 exclusively formed an α-hydroxylated fatty acid product from palmitic acid, but showed that BS_β formed a proportion of decarboxylated 1-pentadecene product (in addition to α- and β-hydroxylated palmitic acid). Aside from OleT (CYP152L1), 1-pentadecene was also detected using in vitro assays and in *E. coli* cell extracts from transformants expressing CYP152-related P450 enzymes from the actinobacteria *Kocuria rhizophila* and *Corynebacterium efficiens,* and from the methane utilizing *Methylobacterium populi* [574]. CYP152B1 and a CYP152-related P450 from *Bacillus clausii* have in common the ability to produce only α-hydroxylated palmitic acid (and no 1-pentadecene), as well as having a glutamine residue at the position corresponding to His85 in OleT. However, BS_β also has a glutamine at this position (Q85) and is able to generate α- and β-hydroxylated palmitic acid, as well as pentadecene [574]. Thus, much work still remains to define structural criteria by which these different peroxygenases can partition activities between fatty acid (α- and β-) hydroxylation and oxidative decarboxylation (Fig. 6.32).

Both P450s BS_β and SP_α have negatively charged residues on the proximal surface of the protein, whereas many other P450s have a positively charged surface that aids recognition of redox partners (that possess a negatively charged interaction region) [577, 578]. A logical conclusion is that this is a further evolutionary adaptation consistent with conversion to a H_2O_2-dependent mechanism in such enzymes. However, Liu et al.

demonstrated that OleT fused (at its C-terminal) to the CYP116B2 reductase (PDOR) module was functional in the NADPH-dependent oxidative decarboxylation of various fatty acids, albeit less effectively than the isolated OleT enzyme operating in peroxygenase mode with H_2O_2. The *E. coli* FLDR/FLD proteins also supported myristic acid decarboxylation to 1-tridecene when reconstituted with OleT and NADPH. Catalase had negligible effect on the proportion of 1-tridecene formed by OleT using the NADPH/redox partner-dependent in vitro systems, but did abolish peroxygenase activity, consistent with redox partners driving OleT catalysis via a 'classical' P450 catalytic cycle [582]. Earlier findings from Girhard et al. had also identified the P450 CYP152A2 ($P450_{CLA}$) from *Clostridium acetobutylicum* as a peroxygenase P450 that binds to fatty acids across a range of chain lengths (C8–C18), as well as to methyl esters of C14, C16 and C18 saturated fatty acids, and as an enzyme which shares a similar substrate specificity profile to P450 BS_β. Activities of both $P450_{CLA}$ and BS_β were driven by NADPH with either *E. coli* FLDR/FLD or the P450 BM3 (CYP102A1) reductase domain [551]. Using either H_2O_2 or NADPH and the heterologous redox partners, the catalytic outcomes were similar, with BS_β producing mainly β-hydroxylated fatty acids from C12–C16 saturated fatty acids, while $P450_{CLA}$ generated an excess of α-hydroxylated fatty acids over the β-hydroxylated forms. Using the BM3 reductase as a partner, an approximately fivefold to sevenfold excess of α-hydroxylated fatty acids were produced [551].

In studies using both P450s BS_β and SP_α, additional catalytic versatility for the peroxygenase P450s was shown using so-called decoy molecules. It is recognized (for all of the bacterial P450 fatty acid peroxygenases) that the fatty acid plays a role as both a substrate and an activator of the reaction itself, through binding of its carboxylate to the conserved arginine side chain [579]. However, short-chain fatty acids are not effective substrates for these enzymes, but short-chain fatty acid (C4–C10) decoy molecules were used effectively to induce formation of a reactive iron–oxo species in P450s BS_β, for example, to enable the epoxidation of styrene and hydroxylation of ethylbenzene in the presence of a heptanoic acid decoy molecule [586]. The stereo-selective epoxidation of styrene using *R*-ibuprofen (producing 63% enantiomeric excess of the *S*-styrene oxide) or *S*-ibuprofen (producing 4% enantiomeric excess of the *R*-styrene oxide) as decoys was also demonstrated with P450 SP_α [587]. A similar phenomenon was demonstrated for P450 BM3 with decoy perfluorinated fatty acids (inert to oxidation by the enzyme); here, using NADPH to drive oxidation of the gaseous alkanes propane and butane to their 2-alcohols, with the efficiency of the coupling of NADPH oxidation to alcohol production increased under high pressure [588]. A similar approach was taken with BM3 to enable oxidation of benzene to phenol [589]. Thus, this type of approach may be applicable for diversifying the range of molecules oxidized by peroxygenase and other (redox partner-dependent) P450 enzymes.

6.3.4.3 Fungal Peroxygenases

While not formally part of the cytochrome P450 superfamily, the cysteine thiolate-coordinated fungal peroxygenases are worthy of mention in context of their high catalytic activity, stability, and versatility [590]. The best known of these enzymes is the chloroperoxidase (CPO) from *Caldariomyces fumago* (an organism also known as *Leptoxyphium fumago*), which has a range of catalytic activities typical of heme-containing peroxidases, catalases, and P450s [591]. CPO is able to catalyze oxidation of halides (such as chloride and bromide) to their hypohalous acids, which then can halogenate organic substrates in a nonspecific manner [590]. CPO was characterized for its involvement in synthesis of the chlorinated fungal metabolite caldariomycin [592, 593]. CPO does have some activity in transferring an oxygen atom from H_2O_2 into activated organic substrates such as *p*-xylene and indole [594, 595]. This type of CPO activity does not extend to nonactivated carbon centers [590]. However, this type of activity is characteristic of the unspecific peroxidases (UPOs), first described in the basidiomycete fungus *Agrocybe aegerita* (AeaUPO), with similar enzymes later

identified in the *Coprinellus radians* (CraUPO) and the *Marasmius rotula* (MroUPO) mushrooms [596–598].

The extracellular UPOs catalyze diverse H_2O_2-dependent oxygenation reactions, often with high efficiency. The hemes are proximally coordinated by an exposed cysteine residue in a conserved Pro–Cys–Pro motif, and form a ferrous–CO complex with absorption maximum in the 445–450-nm range, consistent with their P450-like cysteine thiolate coordination [590, 599]. The UPOs are heavily glycosylated, and the AeaUPO crystal structure reveals a magnesium ion close to a heme propionate (its binding involving a Glu–Gly–Asp motif) and a disulfide bridge in the C-terminal region of the enzyme. Charged residues (Arg189 and Glu 186 in AeaUPO) are implicated in peroxide cleavage, and a pentad of phenylalanine residues in the active site is involved in substrate selectivity and binding [600, 601]. The AeaUPO oxidizes a range of aromatic compounds, including toluene and nitrotoluene in successive reactions through their alcohols and aldehydes to the final benzoic acid products [602]. Other substrates for monooxygenation by the AeaUPO and CraUPO enzymes include human drugs such as ibuprofen, naproxen and phenacetin, with reaction outcomes including those typical of P450s (e.g., hydroxylation of aliphatic side chains and aromatic rings, as well as O- and N-dealkylations). Reactions with labeled ($H_2{}^{18}O_2$) peroxide also clearly demonstrated the transfer of ^{18}O atoms into tolbutamide, carbamazepine, and acetanilide substrates, confirming the peroxygenase mechanism and pointing to further biotechnological applications for the UPO enzymes [603, 604]. Other reactions demonstrated for the AeaUPO include oxidation of pyridine, naphthalene, and alkanes, and transient kinetic studies using the peroxide donor m-CPBA demonstrated the formation of a highly reactive heme-thiolate compound I, consistent with a P450-like peroxygenase mechanism [605, 606]. The fungal UPOs thus have important similarities to the P450s, and likely distinct advantages for performing selected reactions, such as alkane hydroxylations [607].

6.3.4.4 Fungal Nitric Oxide Reductases

P450nor (CYP55A1) from *Fusarium oxysporum* (the same organism that produces the BM3-like fatty acid hydroxylase P450foxy) does not catalyze substrate oxidation, but instead performs a reductive reaction leading to the formation of dinitrogen oxide (N_2O or nitrous oxide) from two molecules of nitrogen monoxide (NO or nitric oxide) bound in the P450 active site, according to the reaction scheme: $2NO + NADH + H^+ \rightarrow N_2O + NAD^+ + H_2O$. There is no requirement for an external redox partner, and instead NADH drives catalysis directly [608]. This is the final stage of a respiratory process in which the nitrate/nitrite inducible P450nor catalyzes the last step in the transformation of nitrate/nitrite into dinitrogen oxide [609]. P450nor is a soluble eukaryotic enzyme, and is more closely related to a number of bacterial P450s (e.g., CYP105 family *Streptomyces* P450s) than to non-CYP55A subfamily eukaryotic P450s [610]. Homologs of P450nor were also cloned/purified from the fungi *Cylindrocarpon tonkinense* and *Trichosporum cutaneum*. In the case of *C. tonkinense,* two distinct P450nor isoforms: P450nor1 (CYP55A2) and nor2 (CYP55A3) were isolated and found to differ in their N-terminal sequence, isoelectric point (5.2 and 4.4, respectively) and preference for NADH versus NADPH, but exhibited indistinguishable UV-visible spectra, with a ferrous–CO complex absorption maximum at 447 nm. P450nor1 only used NADH as electron donor (K_m = 320 μM in steady-state reactions), whereas P450nor2 used both cofactors, with K_m values of 320 μM (NADPH) and 710 μM (NADH) [611]. A key difference between the two *C. tonkinense* genes is that *CYP55A2* has a mitochondrial targeting signal sequence, which is lacking from *CYP55A3*. The *F. oxysporum* CYP55A1 also has different isoforms (P450norA and B), but in this case they are generated from a single *CYP55A1* gene which is either translated from the first available initiation codon (that then incorporates a mitochondrial targeting sequence for P450norA), or from the second initiation codon that is located after the targeting sequence, and thus produces the cytoplasmic P450norB version [612]. The *T. cutaneum* P450nor (CYP55A4) has

its ferrous–CO complex maximum at 448 nm, with similar activity levels with either NADH (12,700 mol NO produced min^{-1}) or NADPH (10,540 min^{-1}) [613]. These spectral properties are comparable with those for the *F. oxysporum* P450nor [45].

The production of N_2O by the *F. oxysporum* P450nor is strongly inhibited by carbon monoxide, oxygen and cyanide, and the noninhibited enzyme catalyzes N_2O production at up to 31,500 min^{-1} [45]. Interrogation of the reaction mechanism indicated that the first molecule of NO binds tightly to the ferric heme iron, forming a ferric–NO complex (with Soret A_{max} at ~431 nm) that is then reduced by NADH to form a specific reaction intermediate with A_{max} at 444 nm, and postulated to be a ferric–hydroxylamine radical complex [610, 614]. This transient intermediate then reacts with the second molecule of NO to yield the N_2O product, with this reaction mechanism supported by computational analysis [615]. The *F. oxysporum* P450nor was also shown to catalyze an unusual co-denitrification reaction, whereby N_2O and N_2 are formed from NO and azide (or ammonia) substrates in a reaction that does not require NAD(P)H as a reductant [616]. For the 'typical' P450nor reaction producing N_2O from two molecules of NO, the preference for NADH over NADPH in CYP55A1 can be explained on the basis of steric hindrance from the side chains of two serine residues in the P450 B′ helix (Ser73 and Ser75), which exclude the 2′-phosphate group of NADPH. The CYP555A1 S75G and S73G/S75G mutants considerably improved the reactivity of the CYP55A1 variants with NADPH [617]. The relatively weak binding of NADH and its various analogs to WT CYP55A1 prevented the crystallization of a complex with the native cofactor that might help identify its binding site and enable further exploration of its mechanism of electron transfer. However, the S73G/S75G mutant was found to improve cofactor binding considerably, enabling the determination of the crystal structure of CYP55A1 in complex with the NADH analog NAAD (nicotinic acid pyridine dinucleotide). The structure revealed conformational adaptations to the binding of NAAD, including the motion of the side chains of two arginine residues (Arg64 and Arg174) to facilitate stabilizing interactions with the NAAD pyrophosphate group. The C4 atom of the nicotinic acid ring moiety of NAAD is located above the heme plane at a distance of only 4.2 Å from the heme iron, and (by comparison with the crystal structure of the NO complex of the WT CYP55A1) immediately adjacent to the nitric oxide (Fig. 6.33) [328, 618]. Thus, structural data indicate that there should be direct reduction of the iron-bound NO molecule by hydride transfer (from the pro-*R* side of NADH in the natural reaction) to form the reactive (likely ferric–hydroxylamine radical) species that then goes on to react with a second molecule of NO to generate the N_2O product (and release water and NAD^+).

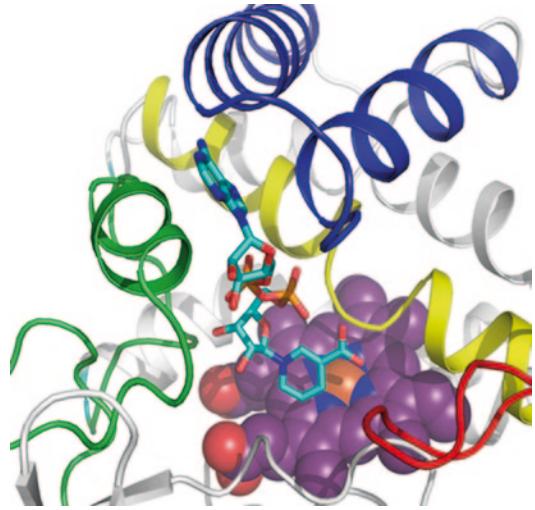

Fig. 6.33 Crystal structure of fungal P450nor (CYP55A1) in complex with NAAD. P450nor catalyzes NADH-dependent reduction of nitric oxide (NO)-bound heme to generate dinitrogen oxide (N_2O). This is an unusual example of a P450 where NADH can access the P450 active site to reduce the substrate-bound heme directly [328]. The image shows the binding mode of the NADH analog nicotinic acid adenine dinucleotide (NAAD) in P450nor, which is consistent with the ability of the enzyme to catalyze stereo-selective hydride transfer from NADH to NO-bound heme (PDB 1XQD) [328]

6.3.5 P450 Interactions with Cytochrome b_5

The eukaryotic membrane-associated cytochromes b_5 (b_5's) are small (typically *ca* 134

amino acids), quite cylindrically shaped proteins that bind *bis*-His coordinated heme and can act as single-electron donors (shuttling their hexacoordinated heme iron between ferrous and ferric states) with electron transfer to/from the heme edge exposed from the protein [116]. The eukaryotic b_5's are integral membrane proteins located on the cytosolic side of the endoplasmic reticulum (attached by a C-terminal hydrophobic domain), and function (along with the FAD-bound partner protein, NADH-cytochrome b_5 reductase) in roles such as electron transfer to desaturase enzymes involved in synthesis of plasmalogens, sterols and unsaturated fatty acids [114]. The b_5's are also known for their influence on catalysis for a number of human and other eukaryotic P450s—altering both enzymatic efficiency and (in some cases) the reaction outcome in terms of products formed. The influence of b_5 in P450-mediated oxidations became evident from pioneering work in the early 1970s, where NADH was found to stimulate NADPH-supported drug metabolism, consistent with the involvement of electron transfer processes involving b_5 reductase and b_5 [114, 619, 620]. Notable examples of the influence of b_5 on P450-dependent oxidation reactions include stimulation of human CYP3A4 metabolism of the anti-cancer drug ellipticine to its therapeutically active 12- and 13-hydroxyellipticine derivatives, which go on to form ellipticine-12-ylium and ellipticine-13-ylium ions that covalently modify DNA [621], and (from studies of human CYP3A4 and b_5 co-expressed in *E. coli*) the enhanced oxidation of both testosterone and nifedipine, in addition to an apparent stabilization of CYP3A4 by the b_5 [622]. A recent report suggests that b_5-dependent stabilization of CYP3A4 expressed in *E. coli* may result primarily from an increase in CYP3A4 messenger RNA (mRNA) half-life mediated by *b5* [623]. In the context of steroid metabolism, the role of b_5 in the reaction chemistry of CYP17A1 is also crucial, with this P450 catalyzing the 17α-hydroxylation of progesterone to 17-hydroxyprogesterone, and of pregnenolone to 17-hydroxypregnenolone. However, 17-hydroxypregnenolone is also further oxidized by CYP17A1 to DHEA in an acyl bond cleavage (17,20-lyase) reaction needed to produce androgens (with the corresponding lyase reaction on 17-hydroxyprogesterone to form androstenedione catalyzed much less efficiently by human CYP17A1) [624]. The lyase reaction is substantially stimulated by cytochrome b_5 proteins that retain a membrane interacting region [625]. While the mechanism by which this process occurs remains controversial, there are compelling data to suggest that the major influence may be conformational (rather than involving electron transfer from b_5 to CYP17A1), with the binding of b_5 inducing a reorientation of a reactive P450 iron–oxo species towards the substrate C20 (to facilitate a lyase reaction path) and away from the C17 position [626, 627].

From a thermodynamic perspective, the midpoint reduction potential (E_m) for the b_5 heme iron Fe^{3+}/Fe^{2+} couple is typically quite positive (e.g., +3 mV vs. NHE for the bovine liver microsomal b_5 and −26 mV vs. NHE for the housefly b_5) [628, 629]. This suggests that any redox role in P450 catalysis could really involve only the second electron delivery, since the heme iron potential for reduction of the ferric substrate-bound forms of the majority of P450s has a considerably more negative reduction potential. For instance, the E_m for the P450 Fe^{3+}/Fe^{2+} couple was determined as −220 mV versus NHE for substrate-free CYP3A4 and −140 mV for the testosterone-bound form in nanodisks [630], and these data are consistent with the requirement of CPR to provide at least the first electron to the P450 heme iron in productive reactions that also involve b_5 [630]. However, the *CPR* gene was successfully disrupted in *Saccharomyces cerevisiae* to generate a strain that still accumulated the sterol ergosterol to ~25 % of the amount found in the parent strain, suggesting that the relevant sterol biosynthesis enzymes (the sterol 14α-demethylase CYP51 and the sterol Δ^{22}-desaturase CYP61) could source electrons for catalysis from another enzyme system in this organism [631]. In further studies using the *Candida albicans* CYP51 P450 with a yeast NADH-cytochrome b_5 reductase (CBR) and b_5 redox system, catalytic activity of the CYP51 was demonstrated in the conversion of 24-methylene-24,25-dihydrolanosterol to its oxidatively demethylated product 4,4-dimethylcholesta-8,14,24(25)-trienol [632]. Subsequently, analysis of the *Phanaerochaete*

chrysosporium CYP5150A2 enzyme revealed that is was able to catalyze hydroxylation of 4-propylybenzoic acid supported by NADPH and CPR from *P. Chrysosporium,* but that the reaction was more efficiently driven by *P. chrysosporium* CBR and b_5 with NADH as electron donor [505]. The *P. chrysosporium* CYP63A2 P450 was also shown to catalyze benzo-a-pyrene monooxygenation with comparable efficiency using either the homologous CPR or CBR/b_5 enzyme systems [633] (Fig. 6.28). Using a mouse genetic approach, Henderson et al. also provided compelling evidence that the CBR/b_5 system can support P450 function, and in this study pointed out the key issue relating to thermodynamics and the unlikely scenario that the b_5 can provide the first electron in the P450 catalytic cycle against a barrier of 200–300 mV. However, they also highlight how a CBR/b_5-driven P450 reaction could occur if this first electron is derived from the CBR (with a redox potential of ~−265 mV vs. NHE), with the second electron being transferred from the b_5 (at ~0 mV) to the oxyferrous form of P450 (with a potential of +20 mV) [634]. Future transient kinetic studies, investigating electron transfer reactions between CBR and b_5 proteins, and their cognate yeast and fungal P450s, should resolve the mechanism of P450 catalysis in these systems.

The cytochromes b_5 are present in lower (e.g., yeasts and fungi) as well as higher eukaryotes, but true b_5-type proteins are rare in prokaryotes. A b_5-like protein (*Ectothiorhodospira vacuolata* cytochrome b_{558}) was identified in this purple phototrophic bacterium, and the protein expressed, purified, and crystallized, leading to the determination of a 1.65 Å structure. This revealed a typical b_5-type fold, with the key difference being the inclusion of a four-residue insertion prior to the histidine sixth ligand to the heme iron, and a disulfide bridge in the protein [635]. A bioinformatics approach also suggested that a distinct b_5 module might be located at the N-terminus of a predicted fatty acid desaturase from the human pathogen *Mycobacterium tuberculosis* strain H37Rv. However, this gene (*Rv3171*) remains uncharacterized [635]. Despite limited evidence for true b_5-like proteins in prokaryotes, there is clear evidence for the interaction of eukaryotic b_5 proteins with bacterial P450 enzymes. For instance, the house fly b_5 binds avidly to the P450 BM3 heme domain (K_d=440 nM), inducing a substrate-like shift in its heme iron spin-state equilibrium towards high spin, suggestive of the induction of a conformational change in the P450. Under anaerobic conditions, the addition of ferrous cytochrome b_5 to ferric BM3 heme domain did not result in P450 reduction (consistent with the large thermodynamic barrier), while the reduced BM3 heme domain and b_5 proteins both remained stable in their ferrous states under anaerobic conditions. However, the introduction of oxygen to this mixture resulted in fast oxidation of both hemoproteins, likely as a consequence of the thermodynamically favorable b_5-dependent reduction of the rapidly formed P450 ferrous–oxy species [636]. In addition, a fluorescently labeled b_5 protein was shown to bind P450cam, with the natural redox partner PD able to displace the b_5 protein—demonstrating an overlapping P450-binding site for these proteins [637]. NMR studies also indicated that b_5 binding to a reduced P450cam–CO complex perturbed many of the same resonances affected by binding of PD, some involving parts of the P450cam structure involved in substrate access and binding orientation. The authors concluded that the primary role of the effector molecule (either PD or b_5 in this case) is to induce formation of/stabilize an 'active' conformation of P450cam that minimizes uncoupling of electron transfer from substrate oxidation during catalysis [638]. Thus, while there is little current evidence for physiological roles of b_5 proteins in bacterial P450 catalysis, there is clearly commonality in the binding mode of b_5 with that of the natural partner proteins for bacterial P450s. Finally, it is interesting to note that the genome sequence of the virus OtV-2 (which infects the unicellular marine green alga *Ostreococcus tauri*) contains a gene predicted to encode a b_5 protein. The gene was expressed in *E. coli*, and the b_5 hemoprotein purified and shown to support lanosterol demethylation by *C. albicans* CYP51 when provided with NADH and the *S. cerevisiae* b_5 reductase enzyme [639].

6.3.6 P450 Electrochemistry

The majority of the P450s have evolved to use NAD(P)H-dependent redox partner systems that deliver two electrons in single-electron steps at different stages of the classical P450 catalytic cycle (Fig. 6.4). However, NADPH (in particular) is an expensive chemical and presents significant cost issues for in vitro P450 turnover studies to produce useful amounts of valuable oxidized products. To make such reactions more cost-effective, an NAD(P)H-regenerating system can be included (e.g., glucose-6-phosphate [G-6-P] and G-6-P dehydrogenase), or reactions can be done in vivo, e.g., using microbial cell cultures [640, 641]. However, there is also continued interest in driving P450 reactions electrocatalytically—either directly at an electrode surface or with the P450s in solution/suspension. Electrochemistry has been used extensively to determine reduction potentials of heme iron in various P450 enzymes, commonly using spectroelectrochemistry, or by cyclic voltammetry or protein film voltammetry methods [581, 642, 643]. However, there have also been several reports of the use of 'direct' electrochemical approaches to drive P450 catalysis—i.e., by providing a source of electrons through an electrode to the P450 (with the protein either in solution or immobilized on an electrode surface), and the outcomes of some of these approaches with microbial and other P450s are detailed below.

In early work in this area, Estabrook and coworkers successfully used electrocatalysis to drive lauric acid hydroxylation through a fusion protein of the rat CYP4A1 with rat CPR (termed rFP4504A1) in aerobic solution. To achieve this, they used a submerged platinum gauze and an applied voltage of −450 mV (vs. NHE), and added the chemical mediator cobalt sepulchrate to carry electrons to the rFP4504A1, cycling through Co^{3+}/Co^{2+} states during its redox reactions with the electrode and the enzyme. The production of 12-hydroxydodecanoic acid was achieved by this system using both the rFP4504A1 fusion enzyme and a mixture of separate rat CYP4A1 and CPR proteins. However, no lauric acid hydroxylation was observed with the CYP4A1 in isolation, pointing to the importance of the reductase interactions with the P450 to achieve effective electron transfer [644]. The application of this methodology was extended to enable electrocatalytically driven hydroxylation of progesterone and pregnenolone using a bovine CYP17A1-CPR fusion protein, 6β-hydroxylation of testosterone and N-demethylation of erythromycin and benzphetamine by a human CYP3A4 fusion and N-demethylation of caffeine and imipramine by a human CYP1A2 fusion. The catalytic rates obtained compared favorably with those achieved using an NADPH-driven system (from ~26% up to 100% as efficient), but were much slower than the rates achieved by either electrocatalysis (110 min^{-1}) or NADPH-driven catalysis (900 min^{-1}) of the natural P450-CPR fusion enzyme flavocytochrome P450 BM3 in its hydroxylation of lauric acid [645]. In studies of drug metabolism using the human P450s CYP2D6 and 2C9, Panicco et al. immobilized these proteins to a gold electrode surface for electrocatalysis, using a chemical 'spacer' method to facilitate covalent attachment of the proteins to a monolayer coating on the electrode, and avoiding direct immobilization on an electrode surface that was shown to denature human P450s [646, 647]. It was found that an immobilized fusion of CYP2C9 to the *Desulfovibrio vulgaris* flavodoxin (FLD) gave an improved electrochemical response over the native CYP2C9, and cyclic voltammetry was used to evaluate the catalytic activity of these P450 systems on the electrode surface in reactions with substrates bufuralol (CYP2D6) and warfarin (CYP2C9-FLD). This type of system has potential applications in high-throughput analyses of drug metabolic reactions that are dependent on individual polymorphisms in these and other human P450s [647].

The issues addressed by Panicco et al. in avoiding P450 denaturation on the electrode surface are important, and there are a number of examples in which the properties of P450 enzymes are markedly different in solution by comparison to those on an electrode surface. For example, determination of the heme iron Fe^{3+}/Fe^{2+} midpoint reduction potential (E_m) in the 1,8-cineole oxidizing P450cin (CYP176A1 from *Citrobacter braakii*)

using cyclic voltammetry indicated that (in the pH range 7–8) the E_m for both cineole-bound and substrate-free forms was ~−50 mV versus NHE and thus not affected by a substrate which induces a considerable shift in the ferric heme iron spin-state equilibrium towards the high-spin state. Solution-state spectroelectrochemistry indicated a rather more negative potential for both forms of the P450 (E_m ~170–180 mV vs. NHE) [648]. However, subsequent spectroelectrochemical studies indicated that there was a substantial difference in the heme iron E_m for the substrate-free (−330 mV vs. NHE) and cineole-bound forms of P450cin (−202 mV vs. NHE), with the extent of increase in the heme iron potential on cineole binding consistent both with the extensive development of high-spin heme iron observed, and with previous such measurements of the relative heme potentials for substrate-free and substrate-bound forms of the well-characterized P450cam and P450 BM3 enzymes [537, 559, 581]. Considerable differences in the E_m for the heme iron Fe^{3+}/Fe^{2+} couple were also observed for the substrate-free P450 BM3 heme domain in solution (−368 mV vs. NHE by spectroelectrochemical titration) and by cyclic voltammetry (~0 mV vs. NHE), with the difference explained through the immobilization of the P450 in a surfactant film in the cyclic voltammetry experiment [581, 649]. In this case, the BM3 heme domain was immobilized in a didodecyldimethylammonium bromide (DDAB) film cast on an edge-plane graphite electrode, and the substantial perturbation to the heme potential was likely caused by partial heme dehydration within the hydrophobic DDAB film on the electrode surface [649, 650]. Similarly, in studies of the *M. tuberculosis* sterol demethylase CYP51B1 immobilized on a graphite surface modified with gold nanoparticles and DDAB, an E_m for the substrate-free P450 Fe^{3+}/Fe^{2+} couple was measured as approximately −43 mV versus NHE, again considerably more positive than that determined by solution-state spectroelectrochemistry at −375 mV versus NHE [21, 651].

Notwithstanding issues with perturbation of heme properties on or near to the surface of an electrode (and associated issues such as P420 formation and poor coupling of electron transfer to substrate oxidation), it remains clear that catalytically competent P450 redox systems can be reconstituted with careful treatment of the P450s and control of the electrode surface environment. Examples of such systems with biotechnological applications include the development of biosensors for the sensitive detection of cocaine, and in which CYP2B4 is immobilized on a carbon electrode [652], and the use of immobilized rat CYP1A1 as an amperometric biosensor for benzo[a]pyrene [653]. P450cam immobilized in a DDAB vesicular system and cross-linked to a glassy carbon electrode has also been applied to electrochemical detection of compounds such as its natural substrate camphor, adamantanone and fenchone [654]. In addition, CYP106A2 from *Bacillus megaterium* (encased in either a cellulose film or in a clay film cast onto the surface of a carbon electrode) was also analyzed by cyclic voltammetry to determine its heme iron midpoint potential (−128 mV vs. NHE) and to analyze influence of prospective substrates, with 4-cholesten-3-one producing a 30-mV positive shift in heme potential (to −98 mV vs. NHE), consistent with its ability to shift the CYP106A1 ferric heme spin-state equilibrium towards high spin [655]. Thus, electrochemistry of microbial (and nonmicrobial) P450s continues to play an important role in the development of the P450 field, both through enabling analysis of the redox properties of P450s, and by providing new routes to P450 catalysis and biotechnological applications for these enzymes.

6.3.7 Nonredox Partner Proteins for Microbial P450s

The *Bacillus* BioI reaction described above (in the section ‘Flavodoxins as bacterial redox partners’) is not the only example of a P450 that has evolved to interact with a carrier protein that acts as the substrate delivery module. In such cases, the carrier protein appears essential for the desired reaction, but does not participate directly in any electron transfer reactions required for production of high-valent iron–oxo species on the heme iron. To date, there are rather few ex-

amples of such nonredox partner proteins recognized for microbial (or other) P450s. In the cases where such proteins have been identified, this has usually been achieved by biochemical analysis, or by analogy with related P450 systems known to use a similar nonredox partner. However, genome sequencing has revealed a number of P450 enzymes fused to potential nonredox partners, as discussed in the section 'Microbial P450-(redox) partner fusion enzymes'. The major examples of involvement of nonredox partner proteins with microbial P450s relate to interactions with substrate delivery modules in the form of carrier proteins, as discussed below and also in the preceding section 'Other biosynthetic actinomycete P450s' under *Microbial diversity of P450s*.

In the case of *Bacillus subtilis* P450 BioI (CYP107H1), the cloning and sequencing of a region of the *B. subtilis* genome containing genes involved in biotin synthesis revealed the presence of the *bioI* CYP gene located at the end of the biotin gene cluster [552]. The BioI protein was purified and shown to be a P450 through spectroscopic analysis, and demonstrated to catalyze fatty acid hydroxylation at mainly the ω-1 to ω-3 positions for myristic (tetradecanoic) acid, and at the ω-1 to ω-5 positions for palmitic (hexadecanoic) acid, with a small amount of the C7 dicarboxylic acid pimelic acid also seen [542, 554]. However, BioI was also shown to be purified from an *E. coli* expression system both as the free protein and in complex with an ACP that was acylated with fatty acids in the size range from C14–C18 [64]. In this BioI–ACP complex, the formation of pimelic acid was demonstrated, likely occurring through three consecutive BioI-dependent oxidations at the C7 and C8 positions on the alkyl chain. This would result in formation of an alcohol, then a vicinal diol and finally C–C bond cleavage using a mechanism analogous to that of the P40scc enzyme involved in the cleavage of the cholesterol side chain in formation of pregnenolone as the primary step in steroidogenesis [555, 656, 657] (Fig. 6.31). Pimeloyl-CoA or pimeloyl-ACP (along with *L*-alanine) is the substrate for the 7-keto-8-amino-pelargonic acid (KAPA) synthase enzyme (BioF) in a central step in the biotin synthesis pathway [656]. The crystal structures of BioI in complex with the small, acidic *E. coli* ACP acylated with three different chain length fatty acids resolved how interaction of the carrier protein influenced the P450-binding mode of the lipids and enabled oxidation at positions distinct from those observed for the non-ACP-bound BioI [131] (Fig. 6.30). The loaded ACP protein interacts with BioI in the active site access area in regions around the β-1 sheet, the loop region between B and B_2 helices and between the F and G helices. A structural comparison between BioI and the P450 BM3 heme domain structure suggests that the B–B_2 and (longer) F–G loop regions in BM3 would clash with ACP and its phospopantetheine linker if bound in a similar mode, and so preclude ACP binding and entry of the substrate [131, 271]. This BioI docking mode enables the ACP to 'feed' the lipid substrate into the active site, which extends beyond the heme to enable the methyl end of the molecule to bend upwards and present the C7–C8 site directly above the heme for oxidative cleavage to occur (Fig. 6.31). The lipid-binding mode is stabilized by several interactions made by the substituent groups of the phosphopantotheine linker, including the phosphate [131]. Thus, clear structural evidence was provided for how specific interactions between BioI and its lipid substrate required for pimelic acid/pimeloyl CoA biosynthesis are provided through substrate delivery by a nonredox partner ACP protein. The processive oxidative reactions required for cleavage of the ACP-bound substrate rely on electron delivery from redox partners, as described in the sections 'Diverse FD partners and flavodoxins as bacterial P450 redox partners'.

BioI is evidently not the only microbial P450 that exploits substrate delivery from a carrier protein as an essential step in its mechanism. There are a number of other P450s now recognized to exploit ACP or PCP modules for the delivery of specific substrates [251]. The CalO2 (CYP248A1) P450 from the actinomycete *Micromonospora echinospora* is involved in the production of the cytotoxic compound calicheamicin (Fig. 6.16), a ten-membered enediyne molecule that binds in the minor groove of DNA and induces radical-mediated DNA strand scission

[230]. CalO2 was postulated to catalyze hydroxylation of the aromatic ring of orsellinic acid as a step in this process, but the failure to reconstitute this activity in vitro along with identification of CalO2 similarity to BioI (33 % identity) led to the theory that the protein required substrate delivery from a carrier protein. The determination of the crystal structure of CalO2 identified that the region between amino acids 54–81 (the B′ and B″ helices) formed a two-helix bundle, and in molecular docking studies the authors found that this structural motif (close to the active site entry region) could provide a docking site for the ACP domain of the CalO5 orsellinic acid synthase protein [230] (Fig. 6.11b). Thus, data collated suggest strongly the involvement of an ACP module in substrate delivery to CalO2, but further work is required to confirm the hypothesis. Other examples of carrier protein-mediated P450 catalysis come with the CYP165 (Oxy) family P450s involved in phenolic coupling (OxyA, B, and E) and aryl carbon coupling (OxyC) reactions in biosynthesis of glycopeptide antibiotics such as vancomycin and teicoplanin [239, 240, 248, 249], and with the β-amino acid hydroxylase OxyD (CYP146A1) that catalyzes production of *L*-b-*R*-hydroxytyrosine [237] for synthesis of the aglycone core of such glycopeptides antibiotics [251] (Fig. 6.19). In each case, these enzymes are considered to oxidize PCP-bound substrates, delivered from PCP modules of an associated nonribosomal peptide synthase (NRPS) enzyme system. A β-amino acid hydroxylase role is also performed by P450 enzymes involved in the synthesis of other antibiotics, including novobiocin (NovI) (Fig. 6.13a) and nikkomycin (NikQ) (Fig. 6.13e), where it has already been shown that carrier protein substrates are competent for oxidation by the relevant P450s [336, 658, 659]. CYP163B3 from *Streptomyces* sp. Acta 2897 also catalyzes β-hydroxylation of three different amino acids in the synthesis of skyllamycin A (an inhibitor of PDGF signaling pathways), forming β-hydroxyphenylalanine, β-hydroxy-OMe-tyrosine and β-hydroxyleucine to be incorporated at different steps in the biosynthetic pathway. Inactivation of the *CYP163B3* gene resulted in formation of a product devoid of β-hydroxylation on any of the amino acids, confirming that P450sky was involved in oxidation of the three different amino acid substrates in separate reactions (Fig. 6.12). It was further shown that the different amino acid substrates were delivered by distinct PCP domains of the skyllamycin NRPS [236]. The Oxy enzymes and P450sky are discussed in more detail in the section ‘Other biosynthetic actinomycete P450s under Microbial diversity of P450s’.

A final protein worthy of a short mention as a potential nonredox P450 partner is the *Saccharomyces cerevisiae* Dap1p, part of a larger family of membrane-associated progesterone receptors (MAPRs). The Dap1p’s have a cytochrome b_5-type heme-binding motif, but lack the histidine residues that provide the axial ligand in b_5. However, yeast Dap1p still binds ferric heme avidly (K_d=400 pM), although has a lower affinity for ferrous heme (K_d=2 μM) [660]. A tyrosine residue (Tyr138) was suggested as a heme ligand, but characterization of a Y138F mutant indicated that the mutant Dap1p still bound ferric heme in a 1:1 ratio with a weakened K_d=200 nM, suggesting that the hydrophobic cavity of Dap1p along with one or more other water or amino acid ligands can stabilize the heme binding [660, 661]. Yeast Dap1p was suggested to have a role in stabilizing the sterol demethylase CYP51 as a result of studies of a yeast *dap1* gene deletion strain that indicated enhanced sensitivity to the DNA-damaging chemical methyl methanesulfonate (MMS) and to fluconazole and itraconazole—antifungal drugs and potent inhibitors of CYP51 [662]. Inclusion of hemin in the growth medium rescued the MMS-sensitive phenotype [663]. However, data remain inconclusive for any specific function in stabilizing CYP51 at the gene or protein level, although Dap1p was also proposed as a potential receptor for P450 enzymes to facilitate their localization to and/or retention in the endoplasmic reticulum [664].

6.4 Microbial P450-(Redox) Partner Fusion Enzymes

As indicated in the previous section, the simplistic view of class I and class II P450 redox systems is now clearly outdated, and a complex collection

of different redox partner systems is now recognized, particularly in lower eukaryotes and bacteria [113, 522]. A number of redox partners are also known to be covalently fused to their cognate P450s—i.e., arise from a gene fusion event that has likely provided a selective advantage to the host organism. These include the intensively studied P450 BM3 (CYP102A1) enzyme from *Bacillus megaterium,* but also a growing number of P450s fused to partners that have no apparent electron delivery role to the P450. The current state of knowledge is discussed below.

6.4.1 Flavocytochrome P450 BM3

P450 BM3 (CYP102A1, BM3), the best known of the P450-redox partner fusion enzymes, is also the enzyme widely considered to be the most catalytically efficient of the known P450 oxidase enzymes—with a catalytic rate constant of ~285 s^{-1} reported for the oxidation of arachidonic acid [665]. It is important here to make the distinction between a 'classical' P450 oxidase that catalyzes reduction of iron-bound dioxygen (O_2) to facilitate monooxygenation through a ferryl–oxo (or compound I) intermediate, and other members of the P450 superfamily that require only the binding of a substrate to initiate catalysis. Such P450s do not activate dioxygen, or use redox partners. Notable examples in this category include mammalian P450s such as thromboxane synthase (CYP5A1) and prostacyclin synthase (CYP8A1) that catalyze molecular rearrangements of their substrates—in the case of the common substrate prostaglandin H_2 forming thromboxane A_2 and prostacyclin (also known as prostaglandin I_2), respectively, and using their heme iron to facilitate homolytic cleavage of the substrate endoperoxide to form the respective products [181, 182, 492]. Another example is the nitrate-/nitrite-inducible *Fusarium oxysporum* P450nor (CYP55A1) which binds NAD(P)H in its active site and uses this to reduce two molecules of nitric oxide (NO) for production of dinitrogen monoxide (N_2O) in an energy-generating pathway. CYP55A1 has a reported rate constant of 1200 s^{-1}at 10°C [614]. Returning to P450 BM3, aspects of its structural and catalytic properties are discussed in the section 'Microbial diversity of P450s', but here we focus on the redox partner apparatus and the electron transfer properties that facilitate its catalytic efficiency.

P450 BM3's structural organization has a typical P450 (heme) domain at the N-terminus (Fig. 6.2b), joined by a peptide linker region to a diflavin (FAD- and FMN-binding) reductase. The reductase is clearly evolutionarily related to the eukaryotic CPR enzymes, but lacks the N-terminal membrane-binding region that tethers the eukaryotic CPRs to the microsomal membrane [666]. Early studies identified BM3 as a *Bacillus megaterium* fatty acid hydroxylase catalyzing hydroxylation of a series of long-chain fatty acids at the ω-1, ω-2, and ω-3 positions (predominantly at ω-2), as well as performing epoxidation reactions on unsaturated fatty acids (Fig. 6.34) [667, 668]. In addition, *CYP102A1* expression was induced in the host organism on addition of barbiturate drugs (including pentobarbital and phenobarbital), enabling the purification of substantial amounts of the flavocytochrome enzyme, and the estimation of a similar V_{max} value of ~4600 min^{-1} for saturated fatty acids from C12–C15 [668, 669]. BM3's high catalytic activity in addition to its convenient single component construction are major factors that have led to its exploitation in various studies aimed at producing novel oxidized molecules through engineering of the P450 active site. Examples include production of oxidized derivatives of steroids, alkanes, and human drugs (Fig. 6.34) [640, 670, 671]. The key to BM3's catalytic proficiency lies mainly in the high rate constants for NADPH-dependent reduction of the FAD cofactor in the CPR domain, and for the internal electron transfer steps from FAD-to-FMN in the CPR and the FMN-to-heme electron transfer. A series of stopped-flow kinetic assays at 25 °C indicated that (at near-saturating NADPH concentrations) the reaction transients for NADPH-dependent flavin reduction in both intact BM3 and its CPR domain were biphasic with rate constants of ~750 s^{-1} (k_{obs} 1) and 130 s^{-1} (k_{obs} 2). The k_{obs} 1 likely reflects the two-electron reduction of the FAD (by hydride ion transfer), while the slower k_{obs} 2 (with a smaller associated absorbance change compared to k_{obs} 1) may be related to an event such as $NADP^+$ dis-

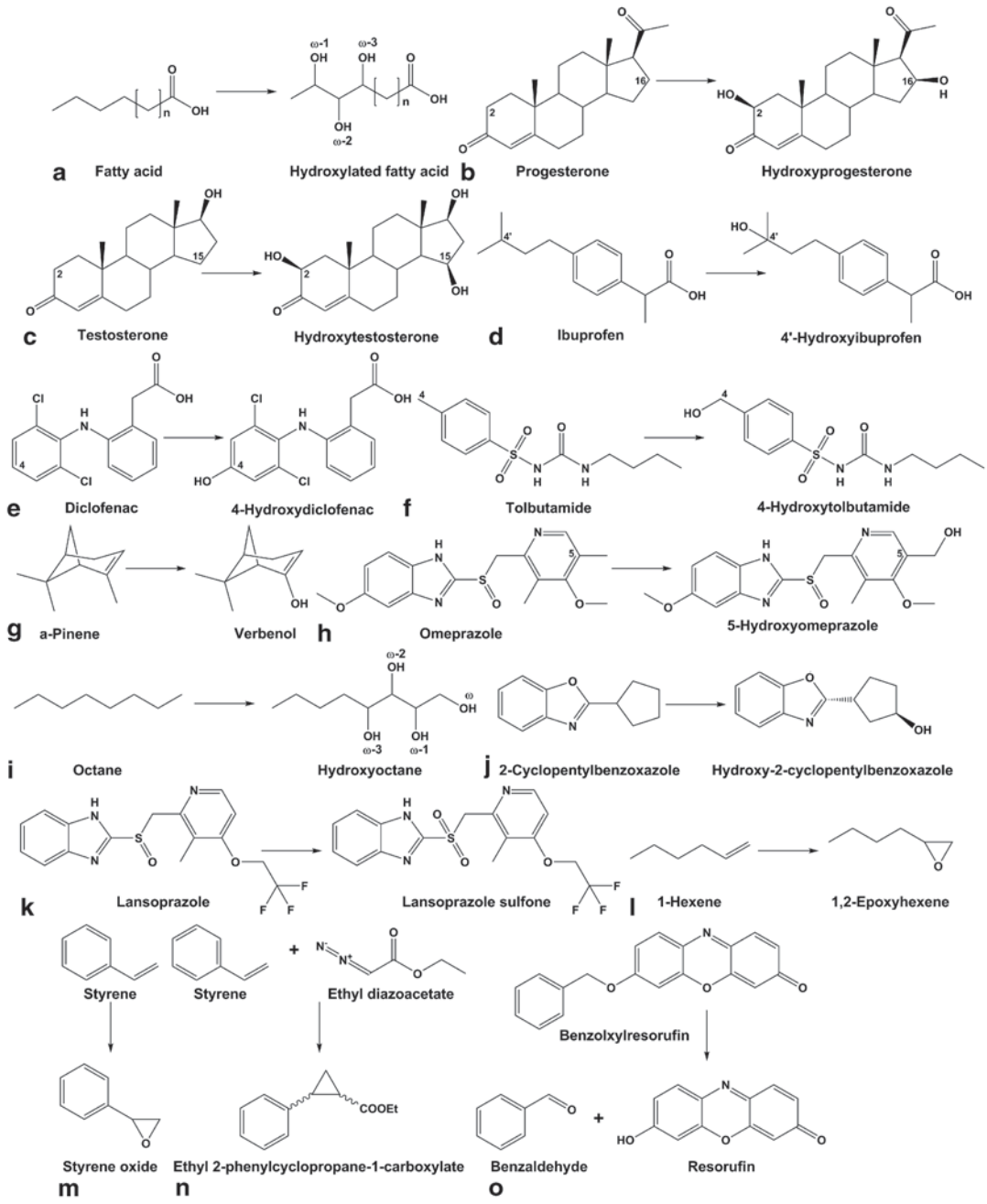

Fig. 6.34 Chemical reactions of wild-type (WT) and engineered forms of flavocytochrome P450 BM3. Examples are shown of substrates and products formed in reactions of the WT and mutant forms of P450 BM3 (BM3). The BM3 enzyme has been extensively engineered using rational, direct evolution and other approaches, and the outcomes highlight the ability of BM3 and its variants to catalyze oxidation of a wide range of chemically diverse substrates. **a** Hydroxylation of the supposed natural substrates for WT BM3, saturated linear chain fatty acids (~C12–C18), at the ω-1, ω-2, and ω-3 positions (indicated) [854]. **b** C2 and C16 hydroxylation of progesterone, and (**c**) C2 and C15 hydroxylation of testosterone, with products observed for both the mono-hydroxy and di-hydroxy forms of testosterone (using BM3 mutants generated by directed evolution) [670]. **d** C2 hydroxylation of ibuprofen, **e** C4′ hydroxylation of diclofenac, and (f) C4 hydroxylation of tolbutamide (using BM3 mutants generated by error-prone PCR) [730]. **g** Conversion of *alpha*-pinene to verbenol by hydroxylation reaction (using a BM3 mutant derived by

sociation or inter-domain conformational reorganization in the CPR. The rate constant for the development of blue flavosemiquinone on the FAD is in excess of 450 s^{-1} under the same conditions, suggesting that the FAD-to-FMN electron transfer is rapid (as might be expected from the close proximity of the FAD and FMN isoalloxazine rings seen in the crystal structure of the rat CPR) and approximately threefold faster than the k_{obs} 2 value [115, 530]. Consistent with this conclusion, the limiting rate constant (k_{lim}) for cytochrome *c* reduction (which occurs exclusively from the FMN cofactor in BM3 and other CPR enzymes) under *pseudo*-first-order conditions using the same temperature/buffer conditions as above was determined as 187 s^{-1} in stopped-flow studies. In addition, the k_{obs} for electron transfer from NADPH to the heme iron in intact BM3 was determined as 223 s^{-1} in CO-saturated buffer and in the presence of near-saturating levels of NADPH and substrate (myristic acid). This experiment involved the 'trapping' of the ferrous, CO-bound complex of the BM3 heme iron (that absorbs maximally close to 450 nm), and thus provides an estimate of how fast the first electron can pass from NADPH through FAD and FMN cofactors in the CPR domain and onto the heme in BM3 [530]. This compares well with the k_{cat} value for arachidonic acid-dependent NADPH oxidation under steady-state conditions, and (given that arachidonic acid is a better substrate than myristic acid for P450 BM3) suggests that the first electron transfer from FMN-to-heme may be a major rate-limiting step in the catalytic process of this enzyme [665]. The electron transfer kinetics of BM3's CPR component are superior to those of their eukaryotic, membrane-associated relatives (e.g., human liver CPR) [672] and BM3 also achieves much faster catalysis that eukaryotic P450 oxidases as a consequence of the covalent linkage of the P450 to the CPR, which promotes productive collisions between the respective domains, as well as by its soluble nature (i.e., not requiring interactions mediated through diffusion and collision of eukaryotic P450s and CPR in microsomal membranes).

The mechanism of electron transfer also differs in BM3 from that of the eukaryotic CPRs—with P450 BM3 going through a 0-2-1-0 electron occupancy in its reductase during catalysis, whereas eukaryotic CPRs use a 1-3-2-1 system. In more detail, many eukaryotic CPRs are isolated in a one-electron reduced, neutral (blue) SQ state—with the SQ stabilized on the FMN cofactor [673]. This is consistent with the structural relationship between this CPR domain and flavodoxins found in microbes, and with the fact that the flavodoxins are well known to stabilize an SQ on their FMN, with the reduction potential for the SQ/HQ couple (E_2) being considerably more negative than that for the oxidized (OX)/SQ couple (E_1). For example, the E_1/E_2 values (relative to the NHE) are −105/−382 mV and −105/−377 mV for the *Bacillus subtilis* YkuN and YkuP flavodoxins, respectively, and −143/−435 mV for the *Desulfovibrio vulgaris* flavodoxin [556, 674]. The relative stabilization of the SQ form arises from factors such as weaker interactions of an aromatic amino acid (typically tyrosine on the *si*-face of the FMN) with the HQ FMN compared to those with the OX and SQ FMN, and from a hydrogen bond from

directed evolution) [855]. **h** C5 hydroxylation of omeprazole (using semi-rationally designed BM3 mutants) [640]. **i** Hydroxylation of alkanes, for example octane at the ω, ω-1, ω-2, and ω-3 positions (indicated in reaction scheme; mutants used obtained from a directed evolution screen) [856–858]. **j** Hydroxylation of an achiral cyclopentanecarboxylic acid derivative (2-cyclopentylbenzoxazole) to the *R, R*-diastereomer (using various mutants of BM3) [859]. **k** Sulfoxidation of the gastric proton pump inhibitor drug lansoprazole to lansoprazole sulfone (using semi-rationally designed BM3 mutants) [860]. **l** Enantiospecific epoxidation of terminal alkenes (e.g., the oxidation of 1-hexene to 1,2-epoxyhexane; using variants from a saturation mutagenesis screen on an existing engineered mutant) [861]. **m** Stereo-selective epoxidation of styrene to styrene oxide (using BM3 mutants from a directed evolution screen) [862]. **n** Carbene transfer from diazoesters (e.g., ethyl diazoacetate) to olefins (e.g., styrene), forming cyclopropane products (e.g., ethyl 2-phenylcyclopropane-1-carboxylate) (using an additional Cys-to-Ser mutation of the heme proximal ligand to improve performance of an existing engineered BM3 mutant) [729]. **o** Oxidative bond cleavage in benzoxylresorufin by WT BM3, leading to production of resorufin and benzaldehyde [863]

an amino acid to the FMN in its SQ state (e.g., a hydrogen bond from the protonated flavin N5 position to the carbonyl group of Gly57 in *Clostridium beijerinckii* flavodoxin) [546, 675, 676]. In eukaryotic CPR, electron donation to the P450 is from the more negative potential FMN HQ, and thus the 'resting' form of the enzyme (in the FMN SQ state) is reduced by two electrons from NADPH to produce first an FAD HQ/FMN SQ form, which is converted to FAD SQ/FMN HQ after inter-flavin electron transfer. The first electron transfer to the ferric P450 heme iron then occurs from the FMN HQ, reducing the heme to the ferrous state and leaving an FAD SQ/FMN SQ form of the reductase. Inter-flavin electron transfer results in an FAD OX/FMN HQ form, which provides the second electron to the P450 (converting the ferric–superoxo species to the ferric–peroxo state), and restores the resting FAD OX/FMN SQ form of the CPR [673, 677, 678]. However, the BM3 mechanism differs in that the heme-reducing species is an FMN anionic SQ, arising due to the unusual structure of the BM3 flavodoxin domain in the FMN-binding region. Specifically, the presence of two basic residues (Lys572 and Lys580) in the vicinity of the pyrimidine ring and flavin N1 could help stabilize an anionic SQ on the FMN and make the FMN SQ/HQ couple more positive, while a shorter, more rigid FMN-binding 'loop' region (by comparison with bacterial flavodoxins) likely prevents its reorientation to facilitate a hydrogen-bonding interaction between protein and a neutral FMN SQ that would stabilize this species. In addition, an amide proton of Asn537 in BM3 hydrogen bonds to the oxidized FMN isoalloxazine N5. Asn547 corresponds to the glycine found in many flavodoxins, but the rigidity of the BM3 FMN-binding loop region makes it unlikely that a reorientation could occur to enable a different hydrogen-bonding interaction between the FMN N5 and the relevant carbonyl oxygen of Asn547 [679]. Murataliev et al. used a combination of transient kinetic analysis and EPR to identify the presence of both FAD (neutral, blue) and FMN (anionic, red) SQs in P450 BM3 on reduction with NADPH, and concluded that heme reduction in BM3 occurred from a one-electron reduced form of the FMN. More extensive reduction of the enzyme by NAD(P)H produced a three-electron reduced form of the BM3 reductase [563]. This form of the enzyme likely corresponds to an 'inactive' form of BM3 identified by Narhi and Fulco through incubation of the enzyme with NADPH in the absence of substrate [668], and redox potentiometry and further investigations, including reduction of the individual FAD- and FMN-binding domains of BM3, suggested that this inactive species has an FMN HQ and a blue SQ FAD [581, 680, 681]. The characterization of the FMN anionic SQ was completed by Hanley and Daff, who established its formation (and disproportionation at ~0.14 s^{-1} at pH 7) in the isolated FMN domain of BM3. The midpoint reduction potential for the FMN oxidized/anionic SQ couple was determined as −240 mV versus NHE, essentially identical to that for the arachidonic acid substrate-bound form of the BM3 heme domain, and thus thermodynamically favorable for electron transfer to the P450 heme iron [562, 581]. In contrast, the redox potential of the SQ/HQ couple of the FMN domain is much more positive at −160 mV (pH 7), making electron transfer to the heme far less favorable and providing a firm thermodynamic basis for the unusual redox cycling process found in BM3.

There have been attempts to recreate efficient BM3-like P450-CPR fusion enzymes by linking the BM3 reductase to other P450s and related enzymes. While this has proved successful in some cases with respect to generating hybrid enzymes that can make useful amounts of products (e.g., ω-hydroxydodecanoic acid in a *Marinobacter aquaeloei* CYP153A-BM3 CPR fusion enzyme) [682], many such chimeras fail to approach the catalytic efficiency of P450 BM3 itself [683, 684]. A likely reason for these observations comes from recent studies that indicate that P450 BM3 may operate mainly as a dimeric form. Black and Martin reported aggregation of intact P450 BM3 from sedimentation velocity and HPLC-size exclusion chromatography experiments, suggesting that BM3 may exist in different forms including monomer, dimer, trimer and higher aggregates [685]. Subsequently, Neeli et al. demonstrated that reconstitution of BM3 fatty acid hydroxylase

activity could be achieved by mixing inactive mutant forms of BM3—specifically a G570D mutant (FMN-deficient) and an A264H mutant (in which the heme is distally coordinated by the His264 imidazole). The recovery of activity in the heterodimer was consistent with the electron transfer from A264H reductase FMN to G570D P450 heme iron—i.e., inter-monomer electron transfer [531]. In later work, Kitazume et al. used cross-linking studies to support the conclusion that dimeric BM3 was the functional form, and from studies of enzyme reactivation in heterodimers concluded that electron transfer from the FAD of one monomer to the FMN of the other (and then to the heme of either monomer) would be consistent with data available [532]. However, subsequent studies revealed that one of the mutants used in the Kitazume et al. study (W1046A in the FAD/NADPH-binding domain of the BM3 reductase) was functional as a fatty acid hydroxylase, and exhibited substantial activity with NADH (as well as NADPH) as the electron donor [686, 687]. This finding leads to reinterpretation of earlier data such that the most likely electron transfer pathway within the BM3 dimer is from NAD(P)H to the FAD of one monomer, and onto the FMN of the same monomer. Thereafter, the FMN may reduce the heme iron of the opposite monomer, and possibly also the heme of the same monomer [686]. A model of inter-monomer (FMN-to-heme) electron transfer in P450 BM3 would be consistent with that proposed for the related eukaryotic flavocytochrome nitric oxide synthase [688] (Fig. 6.35). Thus, by attaching the BM3 reductase to a heterologous P450 enzyme, the catalytic efficiency associated with the structural arrangement of the CPR and P450 domains in the BM3 dimer may be lost, leading to extensive uncoupling of electron transfer to the heterologous P450 and much diminished product formation.

While BM3 has become a paradigm in the P450 superfamily, it is by no means the only representative of this class of P450-CPR fusion enzymes. A large number of homologs are found in other *Bacillus* species, e.g., two in *Bacillus subtilis* [689] as well as other family members in, e.g., *B. licheniformis* (CYP102A7) and *B. cereus* (CYP102A5) [690, 691], and in *Streptomyces avermitilis* (CYP102D1) [692]. In the case of CYP102A7, the enzyme's ability to catalyze oxidation of cyclic and acyclic terpenes points to activities distinct from fatty acid oxidation for CYP102 enzymes in nature. Homologs are also found in the genomes of many eukaryotes, including the model fungal organism *Aspergillus nidulans* and also in strains of *Fusarium oxysporum,* which include plant pathogens. Indeed, the best characterized of the eukaryotic relatives of BM3 is P450foxy (CYP505A1) from *F. oxysporum* MT-811, which is membrane-associated and (like BM3) catalyzes hydroxylation of fatty acids at ω-1 to ω-3 positions, although with activity observed towards shorter chain saturated fatty acids than those preferred by BM3, and exhibiting

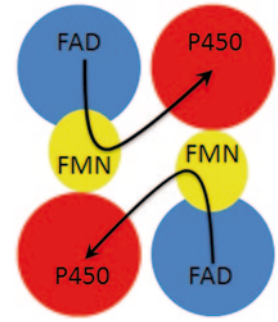

Fig. 6.35 Electron transport in the P450 BM3 dimer. A model is shown for the proposed route of electron transfer in the flavocytochrome P450 BM3 enzyme. Hydrodynamic studies and analyses using engineered P450 BM3 variants indicate that the dimeric form is likely to the catalytically relevant state of the enzyme. In the model, the individual FAD/NADPH (*blue*), FMN- (*yellow*) and heme- (*red*) binding domains of the enzyme are shown aligned antiparallel with one another. Following hydride transfer from NADPH to the FAD, electron transfer is proposed to occur to the FMN in the same monomer, and then to the heme in the opposite monomer, with the FMN-binding domains moving between the FAD/NADPH and heme domains to transport electrons. At present, FMN-to-heme electron transfer within one monomer cannot be ruled out, although recovery of BM3 enzymatic activity in heterodimers formed from inactive monomers that have either (i) FMN binding abolished or (ii) heme inactivated by coordination from an endogenous amino acid side-chain point strongly to the inter-monomer electron transfer model shown [531, 532, 685, 686]. NADPH nicotinamide adenine dinucleotide phosphate, FMN flavin mononucleotide, FAD flavin adenine dinucleotide

substrate inhibition of fatty acid hydroxylase activity for fatty acids of carbon chain length C13 and over [693, 694].

6.4.2 The CYP116B Enzymes

While P450 BM3 is representative of a novel class of P450 enzymes (i.e., a fusion of a soluble P450 to a soluble CPR), the redox partner type was already well known from studies of the mammalian and other eukaryotic CPRs (e.g., [677]). However, the genome analysis studies of De Mot and Parret revealed a completely new type of P450 redox partner system fused to P450s in *Burkholderia* spp. and in the heavy metal tolerant *Cupriavidus metallidurans,* while Roberts et al. expressed in *E. coli* a further member of this class of enzyme from a *Rhodococcus* sp., and showed that it had characteristics of a P450 (in forming a ferrous–CO complex with an A_{max} at ~450 nm in a difference spectrum) and that 7-ethoxycoumarin (7-EC) dealkylation was catalyzed in the P450-containing extract [695, 696]. The organization of this new type of catalytically self-sufficient P450 is with a soluble P450 at the N-terminus and a reductase module resembling a PDOR at the C-terminal [695]. The PDOR module was predicted to contain binding sites for NAD(P)H, FMN, and a 2Fe–2S cluster, and (by analogy with microbial PDOR enzymes) to transport electrons from NAD(P)H to the P450 through electron transfer to the FMN and then through the 2Fe–2S cluster to the heme iron of the fused P450 [697]. In further studies of the *Rhodococcus* enzyme (P450 RhF—formally classified as CYP116B2), a strong preference for NADPH over NADH was established using stopped-flow methods (K_d values of 6.6 μM [NADPH] and 3.7 mM [NADH]). Rapid NADPH-dependent reduction of the electron acceptor potassium ferricyanide was catalyzed by CYP116B2 (k_{cat}=39 s^{-1}), although P450-dependent oxidation of 7-EC was much slower (k_{cat}=4.9 min^{-1}), likely reflecting the nonphysiological nature of this substrate [698]. Further analysis of recombinantly expressed CYP116B2 revealed sub-stoichiometric incorporation of heme and iron–sulfur (2Fe–2S) clusters, which could be enhanced by supplementation of medium with the heme precursor delta-aminolevulinic acid, and by anaerobic incubation of dithiothreitol-treated enzyme with ferrous iron and inorganic sulfide to improve loading of the 2Fe–2S cluster.

The genetic dissection of CYP116B2 enabled the production of the individual heme (P450), PDOR (FMN-FeS), FMN-, and FeS 'domains' of the enzyme, as well as a P450-FMN domain construct [142], in a similar approach as that used for production of the component heme, CPR (FAD-FMN), heme-FMN, and FAD- and FMN-binding domains of P450 BM3 [581, 679, 699, 700]. In both BM3 and the CYP116B family P450-PDOR fusion enzymes, these studies have facilitated the analysis of the thermodynamic and spectroscopic properties of component cofactors in isolation, as well as the kinetic properties of the isolated domains [142], and (in the case of BM3) their structural properties from crystallographic studies [679, 701, 702]. EPR data for CYP116B2 confirmed the presence of a homogeneous 2Fe–2S cluster with *g*-values similar to those of PDOR, and was confirmatory of the successful chemical reconstitution of the cofactor [142]. The midpoint reduction potential (E_m) of the substrate-free CYP116B2 heme cofactor in its isolated domain (−423 mV vs. NHE for the Fe^{3+}/Fe^{2+} redox couple) is considerably more negative than those of the redox couples of the FMN and 2Fe–2S cofactors, and points to the requirement for binding of appropriate substrate(s) to form high-spin ferric heme iron and to increase the heme iron potential to a sufficient extent that electron transfer from the PDOR domain is favored [142]. In the BM3 and P450cam enzymes, the binding of substrates (arachidonic acid and camphor, respectively) leads to extensive conversion of the P450 heme iron from low spin to high spin, concomitant with an increase in the heme iron Fe^{3+}/Fe^{2+} redox couple by ~130–140 mV in both enzymes [537, 581]. In the case of CYP116B2, a similar influence of the physiological substrate on the heme iron potential would be expected. More recent expression and isolation of the CYP116B1 enzyme from *C. Metallidurans* enabled spectroelectrochemical studies

that revealed a rather more positive heme iron E_m of ~−300 mV versus NHE, with the 2Fe–2S cluster and FMN being reduced in the same phase of the redox titration, and with an E_m of ~−160 mV, again pointing to necessity for substrate binding to the P450 to perturb heme iron potential and facilitate inter-domain electron transfer. Transient kinetic studies again indicated that NADPH was the preferred cofactor in CYP116B1, with apparent limiting rate constants for reduction of the PDOR domain being 72 s^{-1} with NADPH, and 22 s^{-1} with NADH. Steady-state analysis also confirmed the much lower affinity for NADH, with K_M values of 3 μM (NADPH) and 102 μM (NADH) in ferricyanide reduction experiments [141]. Preceding studies by Nagy et al. identified a stand-alone P450 enzyme (ThcB, or CYP116A1) involved in the oxidative (N-)dealkylation of the thiocarbamate herbicide EPTC (S-ethyl dipropylthiocarbamate) in *Rhodococcus* sp. strain NI86/21, with the *CYP116A1* gene chromosomally adjacent to its redox partner genes (a 2Fe–2S ferredoxin and a flavoprotein FDR) [139]. CYP116A1 has >50% amino acid sequence identity with the P450 domains of CYP116B1 and CYP116B2, hence their classification in the same P450 gene family. In view of the level of similarity, EPTC and the related vernolate thiocarbamates were also tested as substrates for CYP116B2. Both were found to be hydroxylated on N-propyl groups, with a proportion of N-dealkylated product also observed in the case of vernolate [141]. Thus, commonality in substrate reactivity remains between the CYP116A1 and CYP116B2 enzymes, despite their differing evolutionary paths.

The CYP116B3 enzyme from *Rhodococcus ruber* DSM 44319 was initially identified as a novel FMN-binding P450–redox partner fusion enzyme with domain organization similar to CYP116B2. CYP116B3 was purified and shown to catalyze NADPH-dependent oxidation of a range of molecules such as naphthalene and fluorene (forming ring hydroxylated products), as well as performing side-chain hydroxylation on compounds such as toluene and ethyl benzene [703]. In subsequent work, a combination of rational mutagenesis and directed evolution was used to generate a library of CYP116B3 variants with increased activities for oxidative demethylation of 7-methoxycoumarin and demethylation of 7-ethoxycoumarin, helping to identify 'hotspots' for further engineering to improve activity and diversify substrate selectivity in CYP116B3 [704].

As with BM3, attempts have been made to fuse the CYP116B-type PDOR reductase to other P450 enzymes in order to create more efficient P450 catalysts. Fusions of the CYP116B2 PDOR domain at the C-terminal of P450cam produced variants (with different inter-domain peptide linker lengths used) able to catalyze 5-*exo*-hydroxylation of *D*-camphor in biotransformations using *E. coli* transformant cells [705]. Analogous strategies have also been used to evaluate the catalytic properties of P450cam active site mutant-CYP116B2 PDOR domain fusions in oxidative transformation of molecules such as diphenylmethane [706]. Using a similar approach with *E. coli* biotransformations, Nodate et al. demonstrated (in addition to generation of a functional P450cam chimera) that (i) a microbial benzoate oxidase P450 (CYP203A)-CYP116B2 PDOR fusion could convert 4-hydroxybenzoate into protocatechuate (3,4-dihydroxybenzoate), and that a hypothetical alkane hydroxylase (P450balk from the alkane-degrading marine bacterium *Alcanivorax borkumensis* SK2)-CYP116B2 PDOR fusion catalyzed hydroxylation of octane to 1-octanol [707]. This form of heterologous P450–PDOR fusion was also exploited to drive catalysis by the P450 PikC, involved in the pikromycin macrolide antibiotic pathway in *Streptomyces venezuelae* (Fig. 6.8). The pikC–PDOR fusion catalyzed the hydroxylation of both the 12-membered ring macrolactone YC-17 (to methymycin/neomethymycin) and the 12-membered ring macrolactone narbomycin to pikromycin in vitro, and with a higher catalytic efficiency than observed using nonfused PikC supported by spinach FD and FDR partners [708]. In more recent work, the same group demonstrates intriguing data for the multifunctional P450 MycG (CYP107E1) from *Micromonospora griseorubida* that catalyzes epoxidation and hydroxylation reactions on 16-membered ring mycinamicin macrolide antibiotics (Fig. 6.17). It was found that

the products formed from a MycG–CYP116B2 PDOR fusion in vitro were the physiologically relevant ones, whereas considerable amounts of novel N-demethylated products were observed when the MycG was reconstituted with separated CYP116B2 PDOR, or a PDOR hybrid formed from the CYP116B2 FMN-binding domain with the native 2Fe–2S domain swapped for the spinach FD sequence [709]. Thus, there have been some notable successes in the generation of novel P450 oxidase biocatalysts using fusions of heterologous microbial P450s to the CYP116B-type reductase module. There are no structural data for these fusion enzymes, and negligible data published for the aggregation states of native or chimeric forms of these proteins. However, a potential reason for the relative success of this type of fusion (compared to heterologous P450 fusions with the BM3 CPR) may be that the system is monomeric and does not suffer from steric hindrance to catalysis that may occur in the BM3-type system due to dimerization (probably occurring through the CPR domain).

6.4.3 P450 Fusions to Flavodoxin and FD Proteins

A small number of P450 fusion protein systems are known in which the P450 is fused to either a flavodoxin (FLD) or a ferredoxin (FD) protein. Such fusions decrease the complexity of a class I-type redox partner system to two components—but still require an FDR-type component for delivery of electrons to the FD/FLD component of the fusion enzyme. The best characterized of these enzymes is the XplA FLD-P450 fusion enzyme (CYP177A1) from *Rhodococcus rhodochrous* strain 11Y, which was identified as a P450 contributing to the breakdown of nitrated explosive molecules, and more specifically hexahydro-1,3,5-trinitro-1,3,5-triazine (known as RDX or Royal Demolition Explosive) [710]. *R. rhodochrous* strain 11Y could degrade RDX when provided with the explosive as a sole source of nitrogen. The CYP177A1 P450 (product of gene *xplA*) was identified as an enzyme responsible, and to be formed from the fusion of a flavodoxin-like protein (at the N-terminal) to a P450. Immediately upstream on the chromosome is the *xplB* gene, encoding an ADR-like protein. Thus, a two-component P450 redox system was identified, involving NAD(P)H-dependent electron transfer to the FAD cofactor in XplB, and then reduction of the FLD in XplA and electron transfer from FMN to heme in XplA [710]. Nitrite was released as an early product of RDX degradation by XplA, suggesting that a denitration mechanism was involved that could lead to destabilization of the product and subsequent ring cleavage to facilitate the complete degradation of the molecule [710, 711]. The metabolite 4-nitro-2,4-diazabutanal (NDAB) was shown to be produced during RDX degradation by *Rhodococcus* sp. strain DN22, and studies with rabbit CYP2B4 also indicated that this P450 produced the same metabolite, as well as two molecules of nitrite per NADPH molecule oxidized when reconstituted with CPR, providing further evidence for the involvement of cytochrome P450 in bacterial RDX degradation [712]. XplA was shown to degrade RDX anaerobically when reconstituted with NADPH and an exogenous FDR, with NADPH oxidation tightly coupled to RDX degradation. In addition, transgenic *Arabidopsis thaliana* plants engineered to express *xplA* depleted RDX when grown in liquid media, and were also resistant to RDX-mediated phytotoxicity when grown in RDX-contaminated soil [134]. The phytoremediation study was extended to show that *A. thaliana* transformed with *xplA, xplB* and the 2,4,6-trinitrotoluene (TNT) degrading *nfsI* nitroreductase from *Enterobacter cloacae* could remove RDX from soil contaminated with RDX and TNT at levels that were inhibitory to plants expressing *xplA* alone. Plants expressing both *xplA* and *xplB* were found to have lower concentrations of RDX in aerial tissues, and thus are potentially less toxic to herbivores [713]. The *xplA*/*xplB* gene pair was found to be distributed widely in *Rhodococcus* sp. strains able to deplete RDX from the medium during aerobic growth, and different RDX degradation pathways were hypothesized, resulting from ring cleavage by hydrolysis following either one (anaerobically) or two successive (aerobically) reductive

denitration steps. Under anaerobic conditions, methylenedinitramine (MEDINA) is formed, along with one molecule of nitrite and two of formaldehyde. Under aerobic conditions, NDAB (along with two molecules of nitrite and one of formaldehyde) is formed [714, 715]. Studies of products formed from RDX degradation by *Rhodococcus* sp. strain DN22 in presence of $^{18}O_2$ or $H_2{}^{18}O_2$ indicated that the denitration step did not involve O_2 or H_2O, but that these molecules are involved in subsequent chemical and biochemical processes, although aspects of the degradation mechanism remain uncertain [136].

The XplA P450 heme domain structure was determined, showing a typical P450-fold with imidazole (retained from purification using nickel affinity chromatography) as a sixth ligand to the heme iron. The nonheme ligated imidazole nitrogen is hydrogen bonded to a water molecule, that is in turn hydrogen bonded to the peptide NH of Ala395 and the peptide carbonyl of Val391, stabilizing its binding in the active site. The P450 acid/alcohol pair (typically Glu or Asp/Thr or Ser) is replaced by Met394/Ala395 in XplA, suggesting that the enzyme has evolved for a predominantly reductive function. An A395T mutation substantially diminished binding affinity of RDX and decreased the catalytic efficiency (k_{cat}/K_m ratio) for RDX degradation by ~200-fold) [135]. Light-scattering studies indicated that the XplA flavocytochrome is monomeric, and imidazole was shown to have an unusually high affinity for XplA (K_d = 1.06 μM), consistent with the stabilized ligation mode observed in the imidazole-bound XplA heme domain crystal structure [135, 137]. Extensive dialysis was done to remove imidazole from XplA and to define its Soret maximum in the ferric, substrate-free state as being at 417 nm, blue-shifted (by ~4 nm) to values in previous reports in which residual imidazole remains bound [137]. FMN binding to the XplA N-terminal flavodoxin (FLD) domain was quantified by fluorimetric titration and revealed a weak K_d (~1.1 μM), almost two orders of magnitude higher than that for many other microbial flavodoxins [137, 556, 716]. Reconstitution of the as-purified XplA with FMN produced a spectrum with much better-defined flavin features, confirming that XplA becomes flavin depleted during purification unless buffers contain additional FMN. RDX binding induces a near-complete high-spin shift in the XplA heme iron, with a K_d of 7.5 μM [137]. Spectroelectrochemical titrations indicated an unusually positive redox potential for the XplA FMN SQ/HQ couple (−172 mV vs. the NHE) compared to most other flavodoxins (e.g., −433 mV vs. NHE for the *E. coli* FLD), likely reflecting an unusual binding mode of the FMN and perhaps consistent with its weak affinity for XplA [137, 549]. The substrate-free XplA heme iron potential is −268 mV versus NHE, but the reductive conversion of the RDX substrate means that it was not possible to obtain a redox potential for the substrate-bound form [137]. However, based on preceding studies of other P450 enzymes and the extensive high-spin conversion of the XplA heme iron on binding RDX, an increase in heme potential of ~130–140 mV might be expected (i.e., perhaps to ~−130 mV vs. NHE at near-saturating RDX). This would suggest that the FMN HQ is the relevant electron donor to the RDX-bound XplA heme, which is also the case for the reduction of eukaryotic P450s by CPR [137, 581, 717].

XplA is clearly a P450 with proven potential for biotechnological applications in bioremediation of explosive contaminated soil, and the *xplA* gene may have evolved over the past ~50 years during which RDX has become a major global pollutant in soil and groundwater. To date, *xplA* genes are restricted to bacteria of the order *Actinomycetales,* suborder *Corynebacterineae* (particularly in *Rhodococci*), and the unusual thermodynamic properties of this enzyme appear to indicate adaptation to favor a mainly reductive function [137, 713].

The only characterized example of a P450-ferredoxin fusion protein is found in the methane oxidizing proteobacterium *Methylococcus capsulatus*. MCCYP51FX was identified through a screen of the genome of *M. capsulatus* for the presence of a sterol demethylase (CYP51 family) P450. The screen revealed a single candidate, with a gene encoding a CYP51-like P450 fused to an FR at the C-terminus via an alanine-rich linker region [545]. The P450–FD fusion is 551

amino acids long and is the only P450 encoded by the bacterium. It shares 49% identity with the *M. tuberculosis* CYP51B1, and its FD portion has 42% identity to the *M. tuberculosis* 3Fe–4S ferredoxin Fdx (product of gene *Rv0763c*) that lies immediately adjacent to *CYP51B1* on the *M. tuberculosis* genome [21, 545]. EPR spectroscopy confirmed a MCCYP51FX thiolate-coordinated heme iron, consistent with the ferrous–CO complex maximum at 448 nm. Binding of lanosterol produced a type II UV-visible difference spectrum, suggestive of inhibitor-like (rather than substrate-like) binding. However, reconstitution of MCCYP51FX with spinach FDR and NADPH produced a 14α-demethylated 4α-methyl-5-α-ergosta-8,14,24(28)-trien-3β-ol product from lanosterol, consistent with a bona fide sterol demethylase, albeit with a rather low rate constant of 0.24 min^{-1} [545]. Further studies are required in this case (as for the *M. tuberculosis* CYP51B1) to establish the physiological function of MCCYP51FX and to determine whether *M. capsulatus* metabolizes sterols.

6.4.4 Other Characterized P450-Partner Fusion Enzymes

A relatively new field in P450 biochemistry relates to the discovery and characterization of P450 enzymes fused naturally to proteins unlikely to play a role in electron donation to the P450. The development of this area has understandably been fuelled by the advent of high-throughput genome sequencing—which has pointed to several new examples of P450-redox partner and P450-nonredox partner fusions in the microbial kingdom.

6.4.4.1 P450-Peroxidase/Dioxygenase Fusion Enzymes

Probably the best examples of P450 enzymes covalently linked to a nonredox partner are the fungal Ppo enzyme(s), which are discussed in the section 'Microbial diversity of P450s' earlier in this chapter. The Ppo's are natural fusions of an N-terminal peroxidase/dioxygenase domain to a C-terminal P450 domain, and were recognized through their involvement in *Aspergillus nidulans* in the synthesis of oleic acid- and linoleic acid-derived oxylipins (psi factors) that regulate the fungal life cycle through controlling the balance between sexual and asexual spore development [459], as well as in formation of mycotoxins [458, 478, 480]. PpoA was shown to oxidize linoleic acid to 8*R*-hydroperoxyoctadecadienoic acid (8*R*-HPODE) through a mechanism involving hydrogen atom abstraction from the fatty acid C8 to produce a carbon-centered radical that reacts with dioxygen [488]. The P450 then isomerizes the peroxidase product to 5,8-dihydroxyoctadecadienoic acid in a molecular rearrangement reaction that has close mechanistic parallels with that catalyzed by, e.g., mammalian CYP5A1 (thromboxane synthase) and plant (e.g., flax CYP74A1) AOS [183, 488, 718]. Gel filtration studies of PpoA indicated an approximately fourfold higher molecular weight of the protein compared to that predicted from its amino acid sequence (~440 kDa compared to 110 kDa), suggesting a tetrameric structure of the enzyme [488]. A combination of EPR and electron nuclear double resonance (ENDOR) spectroscopy was used to identify a low-spin thiolate-ligated heme in the PpoA P450 domain, and to characterize axial histidine ligation of heme in the peroxidase domain [719]. The *A. nidulans* PpoA (CYP6000C1) was predicted to have a similar domain structure to PpoA, but lacks the phylogenetically conserved cysteine in the P450 domain (replaced by a glycine). The purified enzyme is thus heme deficient, due in large part to the P450 domain being an apoprotein. A G1039C mutation reinstating a cysteine did not restore heme-binding/isomerase activity [17]. PpoC was shown to catalyze dioxygenation of linoleic acid to produce 10-HPODE, but this was not further isomerized. However, 10-HPODE was converted into 10-keto-octadecadienoic acid and 10-hydroxy-octadecadienoic acid, and also decomposed to 10-octadecynoic acid and to volatile C8 alcohols and other products (e.g., 2-octen-1-ol, 1-octen-3-ol, 2-octenal, and 3-otenone). PpoA and PpoC could also catalyze conversion of 8-HPODE and 10-HPODE into their respective epoxy alcohols: 12,13-epoxy-8-hydroxyoctadecenoic acid and 12,13-epoxy-

10-hydroxyoctadecenoic acid, respectively. The P450 domain is not responsible for the formation of the epoxy alcohols [17]. To date, there is no report of the characterization of the PpoB protein.

The first fungal AOS was discovered in *A. terreus,* with linoleic acid oxidized sequentially to HPODE, and then to the allene oxide 9*R*-(10)-epoxy-11,(12*Z*)-octadecadienoic acid (9*R*(10)-EODE). The AOS activity was found to reside in the P450 domain of a Ppo-type peroxidase/dioxygenase-P450 fusion protein. However, the 9*R*-dioxygenase activity was not assigned to a particular enzyme [720]. Subsequent studies identified the requisite activities for allene oxide synthesis in a single peroxidase/dioxygenase-P450 fusion protein from the plant pathogen *Fusarium oxysporum* [721]. Future work on these systems will likely be in the areas of protein crystallography and the analysis of a higher-order structure of the Ppo-type fusion enzymes. Such studies will be important in understanding enzymatic mechanism and roles of active sites residues in both the peroxidase/dioxygenase and P450 domains, and in rationalizing how substrates and product are channelled between these domains.

6.4.4.2 A P450-Hydrolase Fusion in Mycophenolic Acid Synthesis

A gene cluster encoding the biosynthetic pathway for mycophenolic acid (MPA) was identified in *Penicillium brevicompactum* [722]. MPA is an important immunosuppressant drug used to prevent organ rejection after transplantation, and also has potential antimicrobial, antiviral and antitumor applications [723]. An unusual P450 fusion was identified through studies to characterize enzymes responsible for conversion of 5-methylorsellinic acid (5-MOA) into 5,7-dihydroxy-4-methylphthalide (DHMP), the first and second characterized pathway intermediates in MPA synthesis [724, 725]. The *mpaDE* gene was shown to encode a fusion protein comprising a cytochrome P450 (MpaD or CYP631B5, N-terminal) and a Zn-dependent hydrolase (MpaE). The *mpaDE* gene was expressed in *Aspergillus nidulans* strain NID211 and these cells were able to produce DHMP (an activity absent in the parent NID211 strain). The P450 (MpaD) component of MpaDE was predicted to catalyze a methyl hydroxylation reaction on 5-MOA to produce 5,7-dihydroxy-4-methylphthalide, followed by a lactonization reaction on this intermediate by the MpaE hydrolase to form DHMP [722]. The *mpaDE* gene fusion is unique to date, although several fungi have orthologs of *mpaD* and *mpaE* genes. In the cases of *Talaromyces stipitatus* and *Phaeosphaeria nodorum,* the *mpaD (CYP631B4* and *CYP631C2,* respectively) and *mpaE* genes are located close to each other, and to polyketide synthase (*pks*) genes that have strong similarity and conserved domain architecture to the *P. brevicompactum mpaC* gene that encodes MpaC involved in making 5-MOA [722]. There are no data available as yet for the biochemical or structural characterization of the purified MpaDE enzyme.

6.4.5 P450-Partner Fusion Enzymes from Database Analysis

While there is clearly potential for the misassignment of P450-partner fusion enzymes (e.g., if a stop codon is missed between adjacent genes), the fact that there are several instances of certain types of such fusion proteins in related microbial genomes (and sometimes in genomes of diverse microbes) gives confidence that these genes encode bona fide P450-fusion enzymes. Aside from the Ppo enzymes, there are currently only sparse data reporting the characterization of P450-non-redox partner fusion enzymes. Nonetheless, bioinformatics tools such as CDART , which interrogates databases for conserved protein domain profiles rather than sequence similarity per se, identify several potential P450-fusion enzymes [726]. Selected examples of novel types of such P450 fusion proteins identified in several microbial and other genomes using bioinformatics approaches include (in protein domain order) (i) a P450 linked to a Dyp-type (heme-binding) peroxidase in an extended polypeptide in alphaproteobacteria, (ii) an isoprenoid biosynthetic protein linked to a P450, (iii) a GTB-type glycosyltransferase linked to a P450, (iv) a Rieske iron–sulfur cluster-binding domain linked to a P450 in proteobacteria, (v) a metal-dependent

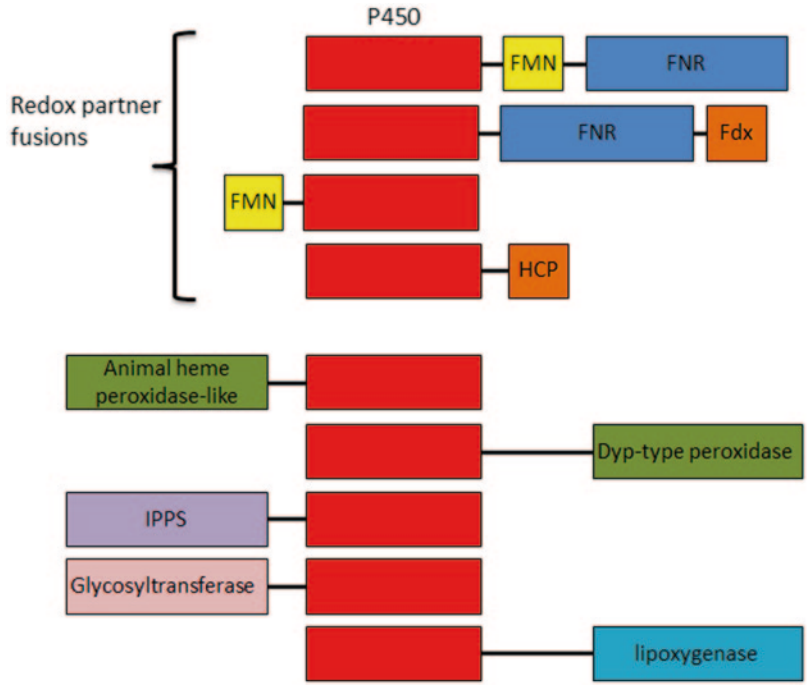

Fig. 6.36 P450 fusion proteins. The figure shows a schematic overview of a number of selected P450-fusion proteins either (i) characterized biochemically or (ii) predicted from analysis of genome sequences. Several such fusion proteins can be classified as redox partner fusions, as typified by the flavocytochrome P450s such as P450 BM3. However, a smaller (but growing) category contains P450 domains fused to other enzyme modules, some containing heme cofactors (e.g., peroxidases). The functions of many such fusions are yet to be established, except in the case of the fungal Ppo proteins. A further category has P450s fused to proteins that are unlikely to have any redox role, and for which the purpose of the fusion protein has yet to be established. From top to bottom, examples shown are (i) P450–CPR fusions of the BM3 type [28], (ii) CYP116B-type P450–phthalate dioxygenase reductase fusions [141], (iii) flavodoxin–P450 fusions of the XplA type [864], (iv) P450-HCP fusions, where HCP indicates a hybrid cluster protein family member. The HCPs contain two iron–sulfur clusters (one of which is a hybrid [4Fe-4S-2O] cluster), and thus are potential P450 redox partners [865];,(v) animal heme peroxidase-type modules fused to P450s [488], (vi) P450s fused to Dyp-type peroxidases [866], (vii) IPPS-type proteins fused to P450s, where IPPS indicates *trans*-isoprenyl diphosphate synthase family member [867], (viii) glycosyltransferases fused to P450s [868], and (ix) P450s fused to lipoxygenases [869]. In examples (iv) to (ix), there is no reported characterization of any member of these P450 fusion classes to date. *FMN* flavin mononucleotide, *FNR* FAD and NAD(P)H-binding reductase module, *Fdx* ferredoxin, *Dyp* dye decolorizing peroxidase

hydrolase linked to a P450 in filamentous fungi, and (vi) a methyl transferase linked to a P450 in filamentous fungi (see Fig. 6.36 for a diagrammatic representation of domain organization in selected P450-partner fusion systems). The growing number of such P450-partner fusion enzymes suggests that they play diverse and important roles in their host organisms, and (like BM3 and Ppo partner fusion P450s) represent evolutionary steps forward to improve catalytic efficiency through enhancement of electron transfer kinetics or (where the fused module is not a redox partner) to consolidate physiologically related activities in a single polypeptide to streamline catalysis through, e.g., more efficient substrate transfer between enzymes.

6.5 Conclusions and Future Prospects

Recent years have seen enormous advances in our understanding of the structure and function of microbial P450 enzymes, as well as an increasing

appreciation of their diversity of catalytic functions and potential as tools for biotechnological applications. There has been a continuous stream of structural data produced for bacterial P450s, aided by the fact that these soluble, cytoplasmic enzymes can often be expressed at high levels in heterologous systems (particularly *E. coli*) and purified efficiently in high yield using affinity tags (particularly His-tags). The development of liquid handling robotics for setting up protein crystallization trials has made the process more efficient and reproducible, and much smaller quantities of protein are now typically required in order to produce crystals of the quality required for structural elucidation. In the past decade, large numbers of new microbial P450 structures have been determined, including several from the human pathogen *Mycobacterium tuberculosis* (including the first sterol demethylase structure for CYP51B1 and high-resolution structures of the cyclodipeptide oxidase CYP121), and the first true structure of an intact P450 membrane protein for the *Saccharomyces cerevisiae* CYP51 [65, 187, 408, 467]. In addition, the crystal structure of the BioI P450 in complex with its ACP partner (which delivers the lipid substrate) is the first true structural representation of a P450 enzyme bound to a partner protein. The structural data in this case demonstrate clearly how the BioI:ACP complex leads to a substrate-binding mode distinct from that which occurs for the interaction of BioI with free fatty acids, and which enables oxidative scission near the center of the substrate to produce a C7 diacid (pimelic acid) for the biotin synthesis pathway [131].

Other major advances in the area of microbial P450 biochemistry have come through the application of high-throughput mutagenesis screening procedures as routes to isolating P450s with novel functions. This is particularly true in the case of directed evolution approaches, where phenotypic screens are used to identify P450 mutants with altered functions, using protein evolution strategies that employ random mutagenesis (sometimes focused on substrate-binding regions of the P450) and/or DNA shuffling/recombination methods. Mutants identified with desired activities are then subjected to further rounds of evolution to further improve their properties [129]. This type of approach has been used particularly successfully in the case of P450 BM3, where several variants with novel substrate selectivity have been generated. These include BM3 mutants with ability to oxidize short-chain alkanes, to perform cyclopropanation reactions (e.g., catalyzing carbene transfer from diazoesters to olefins in *E. coli* cells) and to catalyze regio- and stereo-selective hydroxylation of steroids [670, 727–729]. However, almost certainly the most important recent breakthrough in cytochrome P450 biochemistry is the definitive characterization of P450 compound I—the highly reactive ferryl–oxo heme porphyrin radical species that is ultimately responsible for hydrogen abstraction from the substrate and C–H bond activation. Rittle and Green used CYP119A1 from the thermophilic archaeon *Sulfolobus acidocaldarius* and produced compound I in ~75 % yield by reacting the ferric, substrate-free enzyme with *m*-chloroperbenzoic acid (*m*-CPBA) at 4 °C. UV-vis, EPR and Mössbauer spectroscopy confirmed compound I formation [13]. In subsequent work by Green's group, P450 compound II (that forms after hydrogen transfer to compound I) was also spectroscopically characterized in the *S. coelicolor* CYP158A2—again using *m*-CPBA to convert the ferric P450 directly to a reactive iron–oxo species (compound 0, the ferric–hydroperoxo form) immediately preceding compound I and compound II in the P450 catalytic cycle (Fig. 6.4) [200].

The above recent highlights in microbial P450 biochemistry beg questions as to where the next tranche of advances in our understanding of microbial P450 biochemistry will occur, and what the major industrial and biotechnological applications of these enzymes will be. While major breakthroughs are difficult to predict, there are several potential avenues for exploitation of the P450 enzymes. As discussed above, the diversification of the substrate selectivity of P450s through directed evolution has produced mutants with useful chemical reactivities, including alkane and steroid oxidation (e.g., [670, 727]). However, parallel work done with the highly active, catalytically self-sufficient flavocytochrome P450 BM3 enzyme has also produced

novel variants with capacity to oxidize human drugs to products the same as those produced by the human P450s in vivo, and this has important applications in view of requirements for safety testing of these metabolites (as well as the parent drugs) from the US Food and Drug Administration (FDA) and other regulatory bodies (e.g., [730, 731]). Lessons learned from crystal structures of P450 BM3 and from the growing database of mutants and their effects on catalysis and structural/conformational properties of the enzyme have also helped in the rational or semi-rational generation of BM3 mutants with useful properties—e.g., for variants that efficiently transform the human drug omeprazole to a hydroxylated product the same as that generated by the major human metabolizer CYP2C19 [640]. A further area of applications for P450s was also highlighted in studies by Arnold's group through the use of the BM3 heme (P450) domain as a sensor protein. Paramagnetic metalloproteins (such as the ferric forms of P450) can be used as sensors in magnetic resonance imaging (MRI), and structure-guided directed evolution was done to produce BM3 heme domain mutants that bind avidly to dopamine and serotonin, and which could be used for in vitro studies of neurotransmitter release [732].

The possibility of exploiting P450s for bioremediation is attractive, and there have been numerous studies that have highlighted the abilities of WT and mutant forms of P450s to oxidize PAHs and other environmental pollutants (e.g., [466, 733]). Advances are expected in this area in the near future, with work on the explosive-degrading P450 enzyme XplA from a *Rhodococcus* strain pointing the way for future applications. XplA catalyzes primarily the reductive denitration of the explosive RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine), and is an unusual example of a flavodoxin-P450 fusion enzyme [134, 137, 715]. Contamination of soil and groundwater in areas where the explosive has been used presents major threats to plants, wildlife, and also to humans. Transgenic *Arabidopsis thaliana* plants expressing the *xplA* gene were shown to degrade RDX when grown in liquid media, and also proved resistant to the toxic effects of RDX when grown in explosive-contaminated soil [134, 715].

In the biofuels area, P450 enzymes may also become important players, particularly in view of depleting oil reserves and the limited number of enzymes to date shown capable of generating hydrocarbons that could be useful as biofuels. The OleT P450 from a *Jeotgalicoccus* sp. was shown to catalyze oxidative decarboxylation of a range of fatty acids to generate their n − 1 alkenes [574, 575]. OleT is an efficient peroxygenase P450 that uses H_2O_2 directly to generate the reactive compound 0 on the OleT heme iron, which then progresses to compound I for catalysis. OleT's current specificity range is for long-chain fatty acids (*ca* C12–C20 and beyond), and thus protein engineering studies will be needed to produce variants that can oxidize slightly shorter fatty acids (e.g., producing 1-octene from nonanoic acid) in order to produce 'drop-in' biofuels that are most compatible with most current automobile engines. There is also interest in the insect CYP4G enzymes, which play important roles in the waterproofing of the insect cuticle using hydrocarbons. RNA interference (RNAi) was used to knockdown either *Drosophila melanogaster* CYP4G1 or CPR, leading to insects deficient in cuticular hydrocarbons. *D. melanogaster* CYP4G1 and house fly CYP4G2 enzymes were also shown to catalyze oxidative decarbonylation of long-chain fatty aldehydes to form the n − 1 alkanes in yeast cells co-expressing CPR [734]. The selectivity of the CYP4G enzymes is for fatty aldehydes of chain lengths ~C22 and above, and thus protein engineering will again be required to generate variants that can produce more volatile alkanes. However, there is a clear need for new routes to biofuel production, and the OleT and CYP4G enzymes offer potential solutions for fuel production from fatty acids and aldehydes.

Most P450 enzymes require electron delivery from NAD(P)H via one or more redox partner enzymes, and there are issues with application of P450s for production of oxidized (and other) chemical products in light of factors such as the expense of NAD(P)H, slow rates of electron transfer and catalysis and uncoupling of elec-

tron transfer from substrate oxidation. There is long-standing interest in the application of electrochemistry to drive P450 reactions, and such methods are attractive in terms of potential cost efficiency. Other applications involve using an electrochemical response from a P450 for molecular recognition—i.e., its use as a biosensor. Key challenges to be addressed include avoiding denaturation of the P450 at the electrode surface, with approaches to stabilizing P450s in the literature including protein encapsulation in polymers at the electrode surface, or covalent attachment of the P450s to a self-assembled monolayer on a gold electrode surface [735]. There have been notable successes, including driving lauric acid hydroxylation by P450 BM3 at ~110 turnovers/min, and also in the development of electrochemical sensors for cocaine (using immobilized CYP2B4) and for human drugs (using CYP3A4) [652, 736]. In the latter case, the P450-bound electrode was used in a microfluidic cell format, with the electrochemical response used to identify and quantify the binding of CYP3A4 substrates such as nifedipine and alosetron [736]. Thus, the ongoing development of electrochemistry technologies with P450s has potential to provide new forms of P450 catalytic devices, as well as sensors that can be used for biomedical and chemical detection applications.

A final technological advance of note in the P450 field is the development and use of nanodisks for the encapsulation and solubilization of eukaryotic P450s and other membrane-bound enzymes. Nanodisks are lipid bilayers contained within an amphipathic helical belt (the membrane scaffold protein, or MSP; Fig. 6.37). The membranous P450s are typically mixed with a nanodisk reconstitution mixture of MSP, palmitoyl-oleoyl-phosphatidylcholine (POPC) and sodium cholate, and the mixture immobilized on an Amberlite resin, which initiates the self-assembly process in which the P450 becomes incorporated into a POPC bilayer (typically ~10 nm in diameter) that is stabilized and solubilized by the encircling MSP belt [520, 737]. The nanodisk technology has not only provided an excellent method for generating water-soluble forms of eukaryotic P450s but also enabled the application

Fig. 6.37 Biotechnological applications of microbial P450s. The image shows current and future biotechnological applications for microbial and other P450 enzymes. Clockwise from the *top*: (i) exploitation of P450s for bioremediation, including in transformed plants, (ii) electrocatalysis using P450s in important reactions without the need for NAD(P)H cofactors and cofactor regeneration, (iii) use of P450s for synthesis of compounds such as antibiotics and drug metabolites, (iv) exploitation of nanodisk technology for detailed biophysical and mechanistic characterization of membrane-bound P450s and partner proteins, (v) use of engineered P450s for challenging oxidative chemical reactions, (vi) development of P450 systems for generation of alkane and alkene biofuels, and (vii) exploitation of P450s as sensor proteins for drugs and other bioactive molecules

of several biophysical methods to interrogate their properties. For instance, the analysis of the resonance Raman spectrum of nanodisk encapsulated human CYP3A4 and its response to binding a range of substrates, and studies of the orientation, depth of binding and lipid interactions made by CYP3A4 using combined experimental and molecular simulation approaches [738, 739]. Nanodisk technology will undoubtedly become a more widely used and powerful tool in the study of fungal, mammalian, and other membrane-bound P450s and redox partners—offering opportunities for, e.g., single protein molecule analysis, analysis of P450–lipid interactions and heme redox potential determination. The ability to analyze P450 membrane proteins in a pseudo-soluble form offers opportunities for innovative

new approaches to studying structural and biochemical properties of these systems.

In conclusion, the past decade of P450 research has seen major advances in our understanding of the structure and mechanism of the microbial (and other) P450s. Genome sequencing projects and high-throughput mutagenesis approaches have provided researchers with a wealth of information on new P450 catalysts, and with the tools for generating P450s able to perform novel chemical transformations. The coming years should see further applications for engineered P450s in areas such as fine chemical synthesis, bioremediation, and molecular sensing. Innovative approaches in these and other areas will help maintain a vibrant field for the exploitation of nature's most versatile catalysts.

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P450s in Plants, Insects, and Their Fungal Pathogens

7

Mary A. Schuler

7.1 Introduction

Cytochrome P450 monooxygenases (P450s) are integral components in pathways producing metabolites important for normal growth and development as well as for adaptive strategies that define biotic interactions, including trophic interactions between plants, insects, mammals, fish, and their respective pathogens. Biosynthetic P450s in these pathways can be considered organism-general (fatty acids, sterols) versus organism-specific with examples of the latter including structural components (plant cell walls, insect cuticle, fungal spore walls), signaling networks (plant oxylipins and gibberellins, insect ecdysteroids, fungal gibberellins), and defense compounds (plant terpenoids, alkaloids, furanocoumarins, glucosinolates, insect cyanogenic glycosides, and pyrrolizidine alkaloids, fungal aflatoxins and trichothecenes). Detoxicative P450s are generally organism-specific and frequently evolved from those with catabolic functions. In the interactions between plants and insect herbivores, the activities of synthetic and detoxicative P450s determine how effectively plants can synthesize toxins impeding the growth of insects and how effectively these herbivores can detoxify toxins present in their food sources and hosts. This chapter attempts to highlight biochemical and structural features of the numerous P450s existing in plants, insects and their fungal pathogens. Because it is impossible to do justice to over 18,000 P450 sequences already annotated in these three species groups, readers are guided to several excellent reviews included in each of the following chapter sections.

M. A. Schuler (✉)
Department of Cell and Developmental Biology, University of Illinois, 161 ERML, 1201 W. Gregory Dr., Urbana, IL 61801, USA
e-mail: maryschu@illinois.edu

7.2 Plant P450s

7.2.1 Gene Counts

With the range of compounds that plant species manufacture estimated at over 200,000 [1], individual plant genomes contain varying but always high numbers of P450 genes. Among some of the vascular plant genomes sequenced to date, there are final counts of 142 P450 genes in *Carica papaya* (papaya), 172 in *Nelumbo nucifera* (sacred lotus), 174 in *Morus notabilis* (mulberry), 225 in *Bracypodium distachyon* (model wild grass), 245 full-length genes in *Arabidopsis thaliana* (mouse ear's cress), 270 in *Lycopersicon esculentum* (tomato), 310 in *Populus trichocarpa* (poplar), 316 in *Vitis vinifera* (grape), 334 in *Oryza sativa* (rice), 337 in *Glycine max* (soybean), and 399 in *Solanum tubersum* (potato) as well as preliminary counts of 318 in *Zea mays* (maize), 368 in *Sorghum bicolor* (sorghum), and 412 in *Jatropha curcas* (barbados nut) [2–10]. Because many of their genomes have not yet been sequenced, genome-wide P450 counts for the medicinal plants

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described later in this chapter section are not yet available.

7.2.2 Prominent Synthetic Pathways

Among the larger classes of specialized metabolites synthesized in plant species, terpenoids represent a hyperdiverse class (more than 40,000 structures) that includes many toxic and repellent molecules (monoterpenes (limonene, myrcene, and pinene), diterpenes (taxadiene and abietadiene), triterpenes (amyrin and avenacin), and sesquiterpene lactones (artemisinin and its modified derivatives)) [11, 12]. Alkaloids represent another large and extremely diverse class (more than 12,000 structures) that includes the pharmaceutically relevant isoquinoline and benzylisoquinoline alkaloids (berbamunine, morphine, codeine), monoterpene indole alkaloids (vinblastine, quinine, strychnine), tropane and nicotine alkaloids (nicotine, scopolamine, atropine), purine alkaloids (caffeine), and alkaloid esters (homoharringtonine) [11, 13, 14]. Phenylpropanoids represent a third very large class (more than 8000 structures) that includes flavonols (quercetin), flavonoids (flavone) that are simple hydroxylated flavanones, anthocyanins (pelargonidin) that are complex hydroxylated, methoxylated, and glucosylated flavanones, isoflavonoids (daidzein, genistein) that are rearranged flavonones [11, 15, 16], methylenedioxyphenyl (MDP) compounds (myristicin, sesamin, safrole) that are monomers and dimers of cinnamyl alcohols and/or cinnamic acids [17], and stilbenes (resveratrol, piceatannol, viniferin) that are multimeric derivatives of cinnamoyl CoA and malonyl CoA [11, 15]. Many subclasses of metabolites exist within the large terpenoid, alkaloid, and phenylpropanoid classes and some "mixed origin" metabolites contain components from several classes.

In addition to these larger classes of plant defense compounds, there are smaller classes produced in just a few plant species. Examples of these include: furanocoumarins (a subclass of phenolics with more than 200 structures) that are derived by the attachment of a furan ring to hydroxycoumarin in either a linear orientation (xanthotoxin, psoralen) or an angular orientation (angelicin, sphondin) [18, 19]; glucosinolates (more than 120 structures) that are thioglucosides derived from Met (aliphatic glucosinolates), Trp (indole glucosinolates), and Phe (benzylglucosinolates) [20, 21]; benzoxazinoids that are hydroxamic acids derived from indole [22]; momilactones that are diterpenoids derived from pimaradiene and stemodene [23]; cyanogenic glucosides that are derived from hydoxynitriles and a variety of protein amino acids (Val, Ile, Leu, Phe, Tyr) and, in *C. papaya,* an unusual nonprotein amino acid (cyclopentenyl glycine) [24].

Beyond these various defense compounds, plants synthesize a wide variety of signaling molecules (oxylipins, brassinosteroids, gibberellins, cytokinins, strictolactones) [25], pigments (chlorophylls, carotenoids) [26], and fatty acids and sterols [27]. In the perspective of this chapter, it is worth noting that the production of many of these plant compounds depends on both chloroplast enzymes, which include a small number of soluble P450s, and endoplasmic reticulum enzymes, which include the bulk of membrane-bound P450s.

7.2.3 Gene Conservations and Divergences

Comparisons among the P450 sequences annotated in completed plant genomes, which are available in a number of recent reviews [5, 8, 28, 29], have indicated that relatively few P450 families and subfamilies exist in all plants. Most of those conserved in vascular plant species occur in single-family clans (CYP51, CYP74, CYP97, CYP701) that have been maintained with small numbers of genes or in particular families/subfamilies within multiple-family clans (CYP71, CYP72, CYP85, CYP86) that have expanded numbers of genes. The expansions of the CYP71 clan, which contains 60% of all sequenced plant P450s, and the CYP85, CYP72, and CYP86 clans, which contain 13, 10, and 9% of all plant P450s, are especially notable [8]. Maintained with some evolutionary constraints due to their important roles in plant physiology, individual

conserved families and subfamilies within these clans are typically associated with sterol synthesis (CYP51G mediating sterol 14α-demethylation, CYP710A mediating sterol C22-desaturation), carotenoid synthesis (CYP97 mediating carotenoid hydroxylations), oxylipin synthesis (CYP74A mediating jasmonic acid formation), gibberellin (GA) synthesis (CYP701A and CYP88A mediating sequential conversions in GA formation), fatty acid synthesis (CYP86, CYP94, CYP77A, CYP703A, CYP704B, and CYP709C mediating fatty acid hydroxylations and carboxylations), phenylpropanoid synthesis (CYP73A, CYP75B, CYP84A, and CYP98A mediating cinnamic acid, flavonoid, ferulate, and shikimate hydroxylations, respectively), brassinosteroid synthesis (CYP85A, CYP90), and strigolactone synthesis (CYP711A likely converting carlactone to 5-deoxy-strigol). In addition, some conserved families and subfamilies are associated with catabolism of plant signaling molecules such as oxylipins (CYP74B), brassinosteroids (CYP734A), abscisic acid (ABA; CYP707A), and cytokinins (CYP735A).

Many of the remaining P450 families and subfamilies within the expanded multiple-family clans provide evidence of the many duplication and divergence events that have allowed for the evolution of chemical defense pathways in particular plants or groups of plants. Examples of the chemical diversities resulting from neofunctionalizations within multiple-family clans include the following:

7.2.3.1 CYP71

Within the CYP71 clan that is associated with metabolism of a wide array of compounds, duplicated and closely related CYP79F1 and CYP79F2 genes in *A. thaliana* code for functions in the conversion of short- and long-chain methionine derivatives to oximes [30–32], CYP79B2 and CYP79B3 genes code for functions in the conversion of tryptophan derivatives to another class of oximes [33, 34], and the CYP79A2 gene codes for functions in the conversion of phenylalanine derivatives to yet another class of oximes [35]. Subsequent modifications of these three classes by duplicated and diverged members of the CYP83 family (CYP83A1, CYP83B1) and branchings in their pathways eventually lead to the production of indole glucosinolates, benzylglucosinolates, and aliphatic glucosinolates [36, 37]. Orthologues of CYP79A2 identified in a number of cyanogenic dicots and monocots [38–42] lead to the production of other types of defense molecules. Examples here include: CYP79A1 in *S. bicolor* that mediates the synthesis of the cyanogenic glucoside dhurrin and the CYP79D subfamily in *Manihot esculenta* (cassava) and *Lotus japonicus* (model legume) that mediates the synthesis of linamarin and lotaustralin. Subsequent to these CYP79-mediated steps, the synthetic pathways in each of these species diverge with the product of *S. bicolor* CYP79A2 converted to a cyanohydrin derivative by CYP71E1 [43] and the products of cassava and lotus CYP79D proteins converted to other end products by different and as-yet-uncharacterized monooxygenases.

Other examples within the CYP71 clan include divergent members of the CYP80 and CYP719 families in *Coptis japonica* (japanese goldthread), *Papaver somniferum* (opium poppy), and *Eschscholzia californica* (california poppy) species involved in the synthesis of benzylisoquinoline alkaloids [13, 14, 44] CYP71A13 and CYP71B15 in *A. thaliana* involved sequentially in camalexin synthesis [45, 46], CYP71AV1 in *Artimesia annua* (sweet wormwood) involved in artemisinin synthesis [47], four CYP71C subfamily members in *Z. mays* involved sequentially in the synthesis of benzoxazinoids [22], CYP99A2 and CYP99A3 in *O. sativa* involved in the synthesis of momilactones [48], and the CYP76M subfamily in *O. sativa* involved in oryzalide and phytocassane syntheses [49–51]. In the process of neofunctionalization within the CYP71 clan, some such as CYP76B6 in *Catharanthus roseus* (madagascar rosy periwinkle) have acquired the ability to mediate sequential conversions in a pathway (e.g., geraniol to 10-oxogeraniol) while their close relatives such as CYP76C4 in *A. thaliana* have maintained the ability to mediate only a single hydroxylation (e.g., geraniol to 10-hydroxygeraniol) [52].

7.2.3.2 CYP72

Within the very large CYP72 clan that tends to be associated with metabolism of hydrophobic compounds, the CYP714 family has evolved a number of subfamilies with species-specific activities. Examples here include: CYP714B1 and CYP714B2 in *O. sativa* that are 13-oxidases involved in the synthesis of bioactive gibberellins (GAs) [53], CYP714D1 in *O. sativa* that is a 16,17-epoxidase inactivating non-13-hydroxylated GAs [54], and CYP714A1 in *A. thaliana* that is a 16-carboxylase inactivating 16,17-dihydro GA_{12} [55]. In contrast with these involved in the modulation of GA levels, CYP714A2 in *A. thaliana* and CYP716D1 in *Stevia rebaudiana* have evolved the ability to 13-hydroxylate *ent*-kaurenoic acid and yield steviol, a natural sweetner [55] rather than a modified gibberellin.

7.2.3.3 CYP85

Within the CYP85 clan that is associated with the synthesis and catabolism of signaling molecules in vascular plants (CYP85, CYP90, and CYP734A for brassinosteroids, CYP88A and CYP701A for GAs, CYP707A for ABA, CYP735A for cytokinins) and with the conservation of many gene families and subfamilies, there are several species-specific neofunctionalizations involved in the synthesis of isoprenoids. These include the apparently conifer-specific CYP720B subfamily that mediates oxygenations on monoterpenes (myrcene, pinenes), sesquiterpenes (farnesene) and diterpenes (abietadienol, abietic acid) and lead to the production of diterpene olefins, alcohols, aldehydes, and resin acids [12, 56, 57] as well as the *Taxus* (yew)-specific CYP725A subfamily that mediates sequential steps in taxadiene and paclitaxel syntheses [58].

7.2.3.4 CYP86

Within the CYP86 clan that contains multiple conserved families and subfamilies associated with various fatty acid oxygenations [27, 59], there are fewer examples of species-specific activities neofunctionalized to create new compounds. Even so, some such as CYP94A5 in *Nicotiana tabacum* (tobacco) and CYP94C1 in *A. thaliana,* have evolved the ability to sequentially oxygenate fatty acids to alcohols, aldehydes, and diacids [60, 61], unlike others in their subfamilies.

7.2.3.5 Others

Even within the most highly conserved single-family clans, neofunctionalizations occur. Examples here include: CYP51H in *Avena sativa* (oat) that has diverged from the CYP51G subfamily members in sterol synthesis to produce a multifunctional β-amyrin hydroxylase and epoxidase in avenacin synthesis [62, 63] as well as CYP701A8 in *O. sativa* that has diverged from the CYP701A subfamily members in GA synthesis to produce an *ent*-cassadiene- and *ent*-sandaracopimaradiene-hydroxylase in oryzalexin synthesis [64].

7.2.4 Functional Characterizations of P450s in Model Plants

Prominent among the model plants whose P450 activities are being characterized are *A. thaliana* (representative dicot) and *O. sativa* (representative monocot). Biochemical analyses of their monooxygenases using bacterial (*Escherichia coli*), yeast (*Saccharomyces cerevisiae, Pichia pastoris*), and insect (*Spodoptera frugiperda*) expression systems [65] have helped define conserved and divergent activities in these species as well as substrate overlaps for related subfamily members. The coupling of this information with phenomics analyses of natural (missense) and synthetic (knockouts, knockdowns, overexpressors) mutants has provided important information on the physiological functions of individual P450s and on the genetic redundancies for their multimember P450 subfamilies. Building on the compilations in several recent reviews [4, 7, 29], the current lists of activities for 73 (of 245) *Arabidopsis* P450s and 35 (of 332) *Oryza* P450s are presented in Tables 7.1 and 7.2 with their associated references.

Beyond simple phylogenetic comparisons, structural predictions of *Arabidopsis* and *Oryza* P450s in conserved subfamilies have indicated that they have varying levels of catalytic site di-

Table 7.1 Functionally defined Arabidopsis thaliana P450s

P450	Activity	Pathway	References
51G1	Obtusifoliol 14α-demethylase	Sterols	[132] [133]
71A13	Conversion of indole-3-acetaldoxime to indole-3-acetonitrile	Camalexin	[45]
71A16	Marneral oxidase	Triterpenes	[112]
71B31	Linalool hydroxylase and epoxidase	Monoterpenes	[134]
71B15	Conversion of cysteine indole-3-acetonitrile and dihydrocamalexic acid to camalexin	Camalexin	[135] [136] [46]
72C1	Degradation of brassinosteroids	Brassinosteroid inactivation	[137] [138]
73A5	Cinnamic acid 4-hydroxylase (*t*-CAH)	Phenylpropanoids	[91] [139]
74A1	Allene oxide synthase (AOS)	Oxylipins	[140]
74B2	Hydroperoxide lyase (HPL)	Oxylipins	[141]
75B1	3′-hydroxylase for narigenin, dihydrokaempferol (F3′H)	Phenylpropanoids	[142]
76B3	Linalool hydroxylase	Monoterpenes	[134]
76C4	Geraniol 8- or 9-hydroxylase	Terpene indole alkaloids	[52]
77A4	Epoxidase and ω-hydroxylase on C18 fatty acids	Fatty acids	[143]
77A6	In-chain hydroxylase on 16-hydroxypalmitate	Fatty acids	[144]
79A2	Conversion of phenylalanine to oxime	Benzylglucosinolates	[35]
79B2	Conversion of tryptophan and analogs to oximes	Indole glucosinolates	[33] [34]
79B3	Conversion of tryptophan to oxime	Indole glucosinolates	[33]
79F1	Mono to hexahomomethionine in synthesis of short- and long-chain aliphatic glucosinolates	Aliphatic glucosinolates	[30] [31] [32]
79F2	Long-chain penta and hexahomomethionine in synthesis of long-chain aliphatic glucosinolates	Aliphatic glucosinolates	[31] [32]
81F1	Conversion of indol-3-ylmethylglucosinolate to 4-hydroxy-I3M and 1-hydroxy-I3M	Glucosinolates	[145]
81F2	Conversion of indol-3-ylmethylglucosinolate to 4-hydroxy-I3M and 1-hydroxy-I3M	Glucosinolates	[146] [147]
81F3	Conversion of indol-3-ylmethylglucosinolate to 4-hydroxy-I3M and 1-hydroxy-I3M	Glucosinolates	[145]
81F4	Conversion of indol-3-ylmethylglucosinolate to 1-hydroxy-I3M	Glucosinolates	[145]
82C2	Hydroxylase for 8-methoxypsoralen		[148]
82C4	Hydroxylase for 8-methoxypsoralen		[148]
82G1	Oxidative degradation of C20 geranyllinalool and C15 nerolidol	Homoterpene volatiles	[149]
83A1	Oxidation of methionine-derived oximes	Aliphatic glucosinolates	[150] [36] [151]
83B1	Oxidation of indole-3-acetaldoxime	Indole glucosinolates	[37] [36] [151]

Table 7.1 (continued)

P450	Activity	Pathway	References
84A1	5-hydroxylase for coniferaldehyde, coniferyl alcohol and ferulic acid (F5H)	Phenylpropanoids	[152] [153] [154]
85A1	C6-oxidase for 6-deoxycastasterone and other steroids	Brassinosteroids	[155] [156]
85A2	C6-oxidase for 6-deoxycastasterone and other steroids	Brassinosteroids	[156] [157] [158]
86A1	ω-hydroxylase for satur. and unsatur. C12 to C18 fatty acids	Fatty acids	[159] [160] [161]
86A2	ω-hydroxylase for satur. and unsatur. C12 to C18 fatty acids	Fatty acids	[162] [160]
86A4	ω-hydroxylase for satur. and unsatur. C12 to C18 fatty acids	Fatty acids	[162] [160] [144]
86A7	ω-hydroxylase for lauric acid	Fatty acids	[162] [160]
86A8	ω-hydroxylase for satur. and unsatur. C12 to C18 fatty acids	Fatty acids	[163] [160]
86B1	ω-hydroxylase for C22-C24 fatty acids	Fatty acids	[164]
88A3	Multifunctional *ent*-kaurenoic acid oxidase	Gibberellins	[71]
88A4	Multifunctional *ent*-kaurenoic acid oxidase	Gibberellins	[71]
89A9	Deformylase on fluorescent chlorophyll catabolites	Chlorophyll breakdown	[165]
90A1	23α-hydroxylase for 6-oxo-cathasterone and cathasterone	Brassinosteroids	[166]
90B1	22α-hydroxylase for campesterol, campestanol and 6-oxo-campestanol	Brassinosteroids	[167] [168]
90C1	23α-hydroxylase for multiple brassinosteroids	Brassinosteroids	[169] [170]
90D1	23α-hydroxylase for multiple brassinosteroids	Brassinosteroids	[169] [170]
94B1	ω-hydroxylase for satur. and oxygenated fatty acids	Fatty acids	[171]
94B3	ω-hydroxylase for satur. and oxygenated fatty acids; conversion of JA-Ile to 12COOH JA-Ile hydroxylase for JA-Val and JA-Phe	Fatty acids JA inactivation	[171] [172] [173] [174]
94C1	ω-hydroxylase and in-chain hydroxylase for satur. C12 and unsatur. C18 fatty acids and 9,10 epoxystearic acid; conversion of JA-Ile to 12COOH-JA-Ile	Fatty acids JA inactivation	[171] [61] [173]
96A4	ω-hydroxylase for satur. C12, C14 fatty acids and oleic acid	Fatty acids	[171]
96A15	mid-chain hydroxylase for alkanes and secondary alcohols	Epidermal waxes	[175]
97A3	β-ring carotene hydroxylase	Carotenoids	[176] [177]
97B3	β-ring carotene hydroxylase	Carotenoids	[178]
97C1	ε-ring carotene hydroxylase	Carotenoids	[176] [177]
98A3	3′-hydroxylase for *p*-coumaryl shikimic/quinic acids (C3′H)	Phenylpropanoids	[179]

Table 7.1 (continued)

P450	Activity	Pathway	References
98A8	Hydroxylase on triferuloylspermidine	Phenolamides	[180]
98A9	Hydroxylase on triferuloylspermidine	Phenolamides	[180]
701A3	Multifunctional *ent*-kaurene oxidase	Gibberellins	[69] [70] [71]
703A2	In-chain hydroxylase for C10-C14 fatty acids	Fatty acids	[127]
704B1	In-chain hydroxylase for C16-C18 fatty acids	Fatty acids	[181]
705A5	Thalian-diol desaturase	Triterpenes	[85]
705A12	Marneral desaturase	Triperpenes	[112]
708A2	Thalianol hydroxylase	Triterpenes	[85]
707A1	8′-hydroxylase for ABA	ABA inactivation	[182] [183]
707A2	8′-hydroxylase for ABA	ABA inactivation	[182] [183]
707A3	8′-hydroxylase for ABA	ABA inactivation	[182] [183]
707A4	8′-hydroxylase for ABA	ABA inactivation	[182] [183]
710A1	C-22 desaturase for β-sitosterol	Sterols	[84] [184]
710A2	C-22 desaturase on 24-*epi*campesterol and β-sitosterol	Sterols	[84]
710A4	C-22 desaturase for β-sitosterol	Sterols	[184]
714A1	Conversion of GA12 to 16-carboxylated GA12	Gibberellin inactivation	[55]
714A2	GA12 12-hydroxylase	Gibberellin inactivation	[55]
734A1	26-hydroxylase for brassinolide and castasterone	Brassinolide inactivation	[185] [186]
735A1	*Trans*-hydroxylase for isopentenyladenine phosphates	Cytokinins	[187]
735A2	*Trans*-hydroxylase for isopentenyladenine phosphates	Cytokinins	[187]

ABA abscisic acid

vergence [29]. Some in the most highly conserved subfamilies common to all plants (e.g., CYP84A and CYP98A mediating ferulate and shikimate hydroxylations in lignan synthesis; CYP86A and CYP86B mediating ω-hydroxylations on medium-chain fatty acids; CYP85A, CYP90B and CY90D mediating C6-oxidations, 22α- and 23α-hydroxylations in brassinosteroid synthesis) retain extremely conserved catalytic sites with few changes in substrate contact residues. Others in less conserved subfamilies particular to dicots or monocots have divergent catalytic sites that handle different substrates in species-specific ways (e.g., *Oryza* CYP81A6 in herbicide metabolism vs. *Arabidopsis* CYP81F in glucosinolate synthesis). And, yet others in different families have convergent catalytic sites that mediate the same hydroxylations despite different predicted binding modes (e.g., *Arabidopsis* CYP90B, *Oryza* CYP90B, and *Oryza* CYP724B mediating 22α-hydroxylations on brassinosteroids).

7.2.5 Functional Characterizations of P450s in Medicinal Plants

Many of the specialized plant defense compounds effective in interactions of plants with bacteria, fungi, insects, and mammals have proven useful as pharmaceuticals and nutraceuticals in the treatment of human diseases. As a result, their biochemical pathways, which in-

Table 7.2 Functionally defined Oryza sativa P450s

CYP	Activity/induction	Pathway	References
71P1	Tryptamine 5-hydroxylase	Serotonin	[188] [189]
71Z6	*Ent*-isokaurene C2-hydroxylase	Oryzalides	[190]
71Z7	*Ent*-cassadiene C2-hydroxylase	Phytocassanes	[190]
72A31	Bispyribac sodium metabolism	Herbicide detoxification	[191]
72A18	Peralogonic acid (ω-1)hydroxylase	Herbicide detoxification	[192]
74A5	Allene oxide synthase	Jasmonic acid	[193]
74E1	9-/13-hydroperoxide lyase	Oxylipins	[194]
74E2	9-/13-hydroperoxide lyase		
75B3	Flavonoid 3′-hydroxylase	Flavonoids	[195]
76M5	*Ent*-sandaracopimaradiene C7β-hydroxylase	Oryzalexins	[50]
76M6	*Ent*-sandaracopimaradiene C9β-hydroxylase	Oryzalexins	[51]
76M7	*Ent*-cassadiene C11α-hydroxylase	Phytocassanes	[49]
76M8	*Ent*-sandaracopimaradiene C7β-hydroxylase	Oryzalexins	[51]
81A6	Bentazon and sulfonylurea metabolism	Herbicide detoxification	[196]
85A1	C6-oxidase for 6-deoxocastasterone and steroids	Brassinosteroids	[197]
88A5	*Ent*-kaurenoic acid oxidase	Gibberellins	[198]
90B2	22α-hydroxylase for campesterol	Brassinosteroids	[199]
90D2	C23 hydroxylase on 22-hydroxylated brassinosteroids	Brassinosteroids	[200]
90D3	C23 hydroxylase on 22-hydroxylated brassinosteroids	Brassinosteroids	[200]
93G2	Flavanone 2-hydroxylase	Flavones	[201]
97A4	β-ring carotene hydroxylase	Carotenoids	[202]
97C2	ε-ring carotene hydroxylase	Carotenoids	[202]
99A3	*Syn*-pimaradiene oxidase	Momilactones	[48] [23]
701A6	*Ent*-kaurene oxidase	Gibberellins	[203]
701A8	C3α hydroxylase on *ent*-sandaracopimaradiene, cassadiene and kaurene	Oryzalexins	[64]
704B2	ω-hydroxylase on C16-C18 fatty acids	Fatty acids	[204]
707A5	ABA 8′-hydroxylase	ABA inactivation	[205]
707A6	ABA 8′-hydroxylase		[206]
714B1	GA 13-oxidase	Gibberellin inactivation	[53]
714B2	GA 13-oxidase	Gibberellin inactivation	[53]
714D1	Epoxidase on non-13-hydroxylated Gas	Gibberellin inactivation	[54]
724B1	22α-hydroxylase for brassinosteroid precursors	Brassinosteroids	[199]
734A2	Conversion of 6-deoxo3DT to 6-deoxo3DT-COOH	Brassinosteroid inactivation	[74]
734A4	Conversion of 6-deoxo3DT to 6-deoxo3DT-COOH	Brassinosteroid inactivation	[74]
734A6	Conversion of 6-deoxo3DT to 6-deoxo3DT-OH and 6-deoxo3DT-CHO	Brassinosteroid inactivation	[74]

Satur saturated, *Unsatur* unsaturated

clude large numbers of P450s, O-methyltransferases (OMTs), N-methyltransferases (NMTs), flavin adenine dinucleotide (FAD)-linked oxidoreductases, nicotinamide adenine dinucleotide phosphate hydrogen (NADPH)-dependent reductases, and the like have been explored with ever-increasing genomic resources. Prominent among the medicinal plants being studied are *Coptis, Papaver,* and *Eschscholzia* species that synthesize the benzylisoquinoline alkaloids berberine, morphine, codeine, thebane, noscapine, papaverine, and sanguinarine, *Berberis stolonifera* (barberry) that synthesizes the bisbenzylisoquinoline alkaloid berbamunine, *C. roseus*

that synthesizes the monoterpene indole alkaloid vinblastine, *Hyoscyamus niger* (henbane), and *N. tabacum* that synthesize tropane and nicotine alkaloids atropine, scopolamine, and nicotine, *Glycyrrhiza uralensis* (licorice) that synthesizes the triterpenoid saponin glycyrrhizin, *Panax ginseng* that synthesizes multiple triterpene ginsenosides, *Taxus baccata* that synthesizes the diterpenoid paclitaxel, and *A. annua* that synthesizes the sesquiterpene lactone artemisinin [11, 13, 14, 66, 67]. Figure 7.1 depicts structural subgroups of 1-benzylisoquinoloine alkaloid, Fig. 7.2 indicates P450-mediated modifications in benzylisoquinoline synthesis and Fig. 7.3 depicts P450-mediated modifications in paclitaxel synthesis. Building on compilations in several recent reviews [14, 44, 68], the current list of activities for medicinal plant P450s characterized using one of the heterologous expression systems mentioned above or newer gene-silencing technologies is presented in Table 7.3.

7.2.6 Unusual Features

7.2.6.1 Reactivities

As previously summarized in Mizutani and Sato [44], many of the aforementioned plant P450s have unusual reactivities. Some are capable of intermolecular C-O phenol coupling (*B. stolonifera* CYP80A1), intramolecular C-C phenol coupling (*C. japonica* CYP80G2, *P. somniferum* CYP719B1), C-C bond cleavage (*C. roseus* CYP72A1, *Ammi majus* (bishop's weed) CYP71AJ1, *Pastinaca sativa* (parsnip) CYP71AJ4), rearrangement of carbon skeletons (*H. niger* CYP80F1), ring rearrangements (*G. echinata* CYP93C2, various CYP88A proteins), methylenedioxy-bridge formation (various CYP719A proteins, *Sesamum indicum* (sesame) CYP81Q1), N-oxidations (various CYP79 proteins), sterol desaturations (various CYP710A proteins, *A. thaliana* CYP705A5), as well as dehydrations and cyclizations in camalexin synthesis (*A. thaliana* CYP71A13, CYP71B15).

Others are capable of mediating sequential modifications in synthetic or detoxicative pathways. Examples here include: many CYP88A and CYP701A subfamily members in GA synthesis [69–72], CYP79 family members in aldoxime synthesis [24], *Pinus taeda* (loblolly pine) and *Picea sitchensis* (sitka spruce) CYP720B proteins in abietic acid and other resin acid syntheses [56, 57, 73], *O. sativa* CYP734A in brassinosteroid inactivation [74], *A. sativa* CYP51H in avenacin synthesis [63], *C. roseus* CYP76B6 in strictosidine synthesis [52], *G. uralensis* CYP88D6 and CYP72A154 in glycyrrhizin synthesis [75, 76], *P. ginseng* CYP716A52 in oleanolic acid synthesis [77], *A. annua* CYP71AV1 in artemisinin synthesis [47], *L. japonicus* CYP71D353 in hydroxybetulinic acid synthesis [78], and the previously mentioned *N. tabacum* CYP94A5 and *A. thaliana* CYP94C1 in fatty acid syntheses [60, 61].

7.2.6.2 Residues

Given the radical nature of catalysis in some of the atypical reactions mentioned above and the use of substrate oxygens in others [44], it is not surprising that substitutions occur in the I-helix residues of some plant P450s. Changes within the conserved (A/G)GX(D/E)TT motif containing the oxygen-activating Thr (underlined) [79] occur in CYP719A proteins catalyzing methylenedioxy-bridge formations and CYP719B1 catalyzing phenol coupling reactions with Leu in place of A/G and Ser in place of *T* [80, 81], CYP93C2 catalyzing aryl migration on flavanone with Ser in place of *T* [82], CYP88A3 and CYP88A4 catalyzing aryl migrations on kaurenoic acid with Ser in place of *T* [71], CYP71A13 and CYP71B15 catalyzing dehydrations and cyclizations of indole 3-aldoxime with Ser in place of *T* [45, 46], CYP79 proteins catalyzing aldoxime synthesis with Ser in place of *T* [24], CYP81Q1 catalyzing methylenedioxy-bridge formation with Ala in place of *T* [83], CYP710A proteins catalyzing sterol C22 desaturation with Ala in place of *T* [84], CYP705A5 catalyzing thalianol-diol desaturation with Ala in place of *T* [85], CYP734A proteins catalyzing sequential brassinosteroid oxygenations with Gln in place of D/E [74], CYP51H catalyzing hydroxylations and epoxidations on β-amyrin with His in place

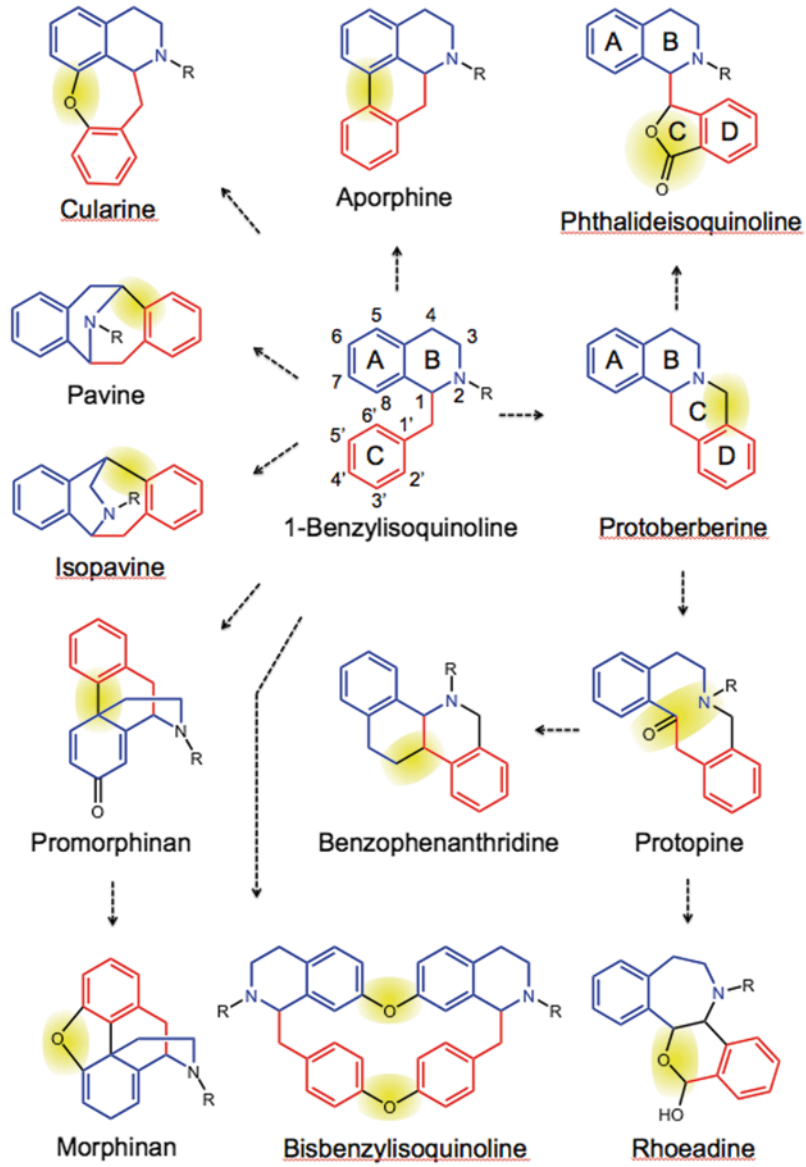

Fig. 7.1 Benzylisoquinoline alkaloid structural subgroups derived from the basic benzylisoquinoline subunit. *Blue* designates the part of each molecule originating from the tetrahydroisoquinoline moiety; *red* designates the part of each molecule originating from the benzylic moiety; *yellow* highlights C–C or C–O bonds formed in the benzylisoquinoline subunit that defines each structural subgroup. Stereochemistry is not indicated since both (*R*)- and (*S*)-configurations exist in many cases. (Excerpted from ref. [14])

of D/E [63], CYP80A1 and CYP80G2 catalyzing phenol coupling reactions on methylcloclaurine and reticuline with Pro in place of A/G [86, 87], and CYP82E4 catalyzing demethylation of nicotine with Asp in place of A/G and Ala in place of G [88].

7.2.6.3 Electron Transfer Partners

Contrasting with the single P450 reductase and cytochrome b_5 sequences present in the vertebrates, higher plant genomes contain multiple P450 reductase (CPR) and cytochrome b_5 (cyt b5) proteins. Phylogenetic analyses of multiple

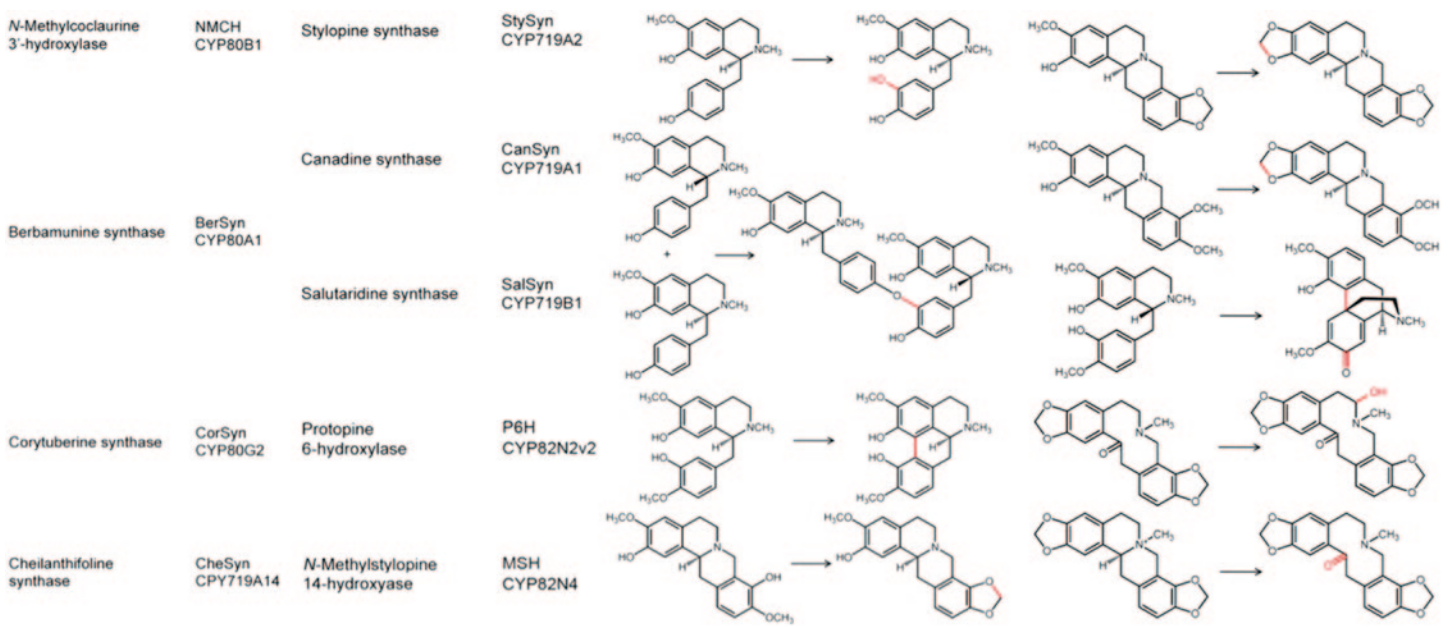

Fig. 7.2 P450-mediated reactions in benzylisoquinoloine alkaloid biosynthesis. Products of P450-mediated reactions in alkaloid syntheses are shown with positions of modifications in *red*. (Excerpted from ref. [14])

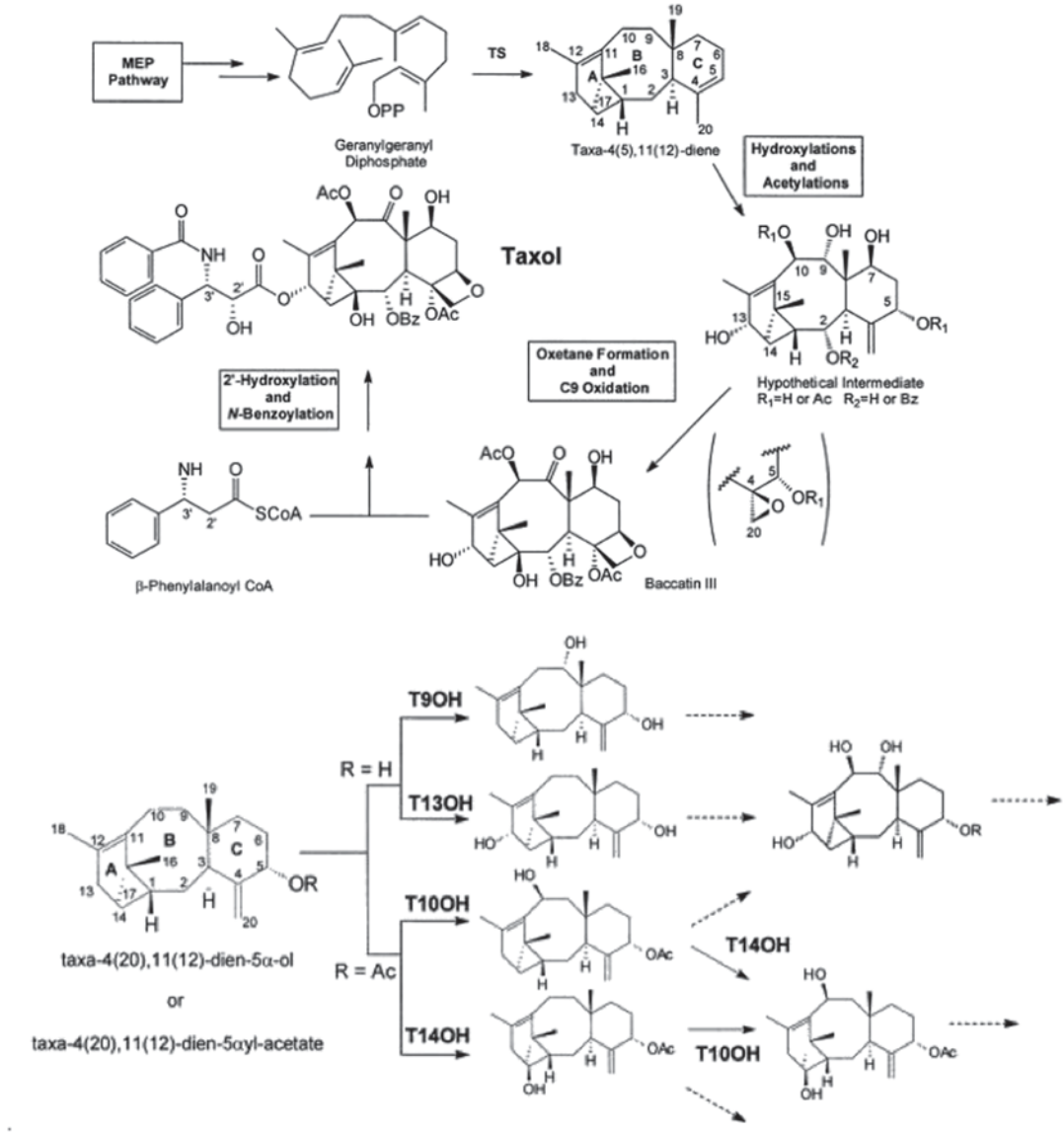

Fig. 7.3 Taxol/paclitaxel biosynthetic pathway. (*Top*) Overview of biosynthetic pathway. (*Bottom*) Bifucation of the taxol biosynthetic pathway following the 5*α*-hydroxylation step showing four taxoid hydroxylations mediated by members of the CYP725A subfamily. The *broken arrows* indicate subsequent undefined metabolic steps. (Excerpted from ref. [58])

Table 7.3 Functionally defined P450s in medicinal plants

Species	CYP	Activity	Pathway	References
Artemisia annua	71AV1	Conversion of amorphadiene to artemisinic acid	Sesquiterpene lactones Artemisinin	[47]
Berberis stolonifera	80A1	Berbamunine synthase	Bisbenzylisoquinoline alkaloids Berbamunine	[86]
Catharanthus roseus	71D12	Taberosine 16-hydroxylase	Terpene indole alkaloids Vindoline	[207]
	71D351	Taberosine 16-hydroxylase	Terpene indole alkaloids Vindoline	[208]
	71BJ1	Taberosine 19-hydroxylase	Terpene indole alkaloids Hörhammericine	[209]
	72A1	Secologanin synthase	Terpene indole alkaloids	[210]
	72A224	7-deoxyloganic acid 7-hydroxylase	Terpene indole alkaloids	[211]
	76B6	Conversion of geraniol to 10-oxo geraniol	Terpene indole alkaloids	[212] [52]
Coptis japonica	80B2	N-methylcoclaurine 3′-hydroxylase	Benzylisoquinoline alkaloids Berberine	[80]
	80G2	Corytuberine synthase	Aporphine alkaloids magnoflorine	[87]
	719A1	Canadine synthase	Protoberberine and phthalideisoquinoline alkaloids Berberine	[80]
Eschscholzia californica	80B1	N-methylcoclaurine 3′-hydroxylase	Benzophenanthridine alkaloids Reticuline	[213]
	82N2	Protopine 6-hydroxylase Allocryptopine 6-hydroxylase	Benzophenanthridine alkaloids Sanguinarine	[214]
	719A2	Stylopine synthase	Protoberberine and benzophenanthridine alkaloids Sanguinarine	[215]
	719A3	Stylopine synthase	Protoberberine and benzophenanthridine alkaloids Sanguinarine	[215]
	719A5	Cheilanthifoline synthase	Benzophenanthridine alkaloids Sanguinarine	[81]
	719A9	Formation of methylenedioxy bridge in reticuline	Pavine alkaloids Californidine	[81]
Glycyrrhiza uralensis	72A154	β-amyrin 30-oxidase	Triterpenoid saponons Glycyrrhizin	[76]
	81E1	Isoflavone 2′-hydroxylase	Hydroxyisoflavones	[216]
	88D6	β-amyrin 11-oxidase	Triterpenoid saponons Glycyrrhizin	[75]
	93B1	Flavanone 2-hydroxylase	Hydroxyflavanones	[217]
	93E3	β-amyrin 24-hydroxylase	Triterpenoid saponons Glycyrrhizin	[75]
Nicotiana tabacum	71D20	5-epiaristolochene 1,3-dihydroxylase	Sesquiterpene Phytoalexins Capsidiol	[218] [121]
	82E4	Nicotine N-demethylase	Nicotine alkaloids	[88]
	82E5	Nicotine N-demethylase	Nicotine alkaloids	[219]
	82E10	Nicotine N-demethylase	Nicotine alkaloids	[220]

Table 7.3 (continued)

Species	CYP	Activity	Pathway	References
Panax ginseng	716A47	Dammarenediol 12-hydroxylase	Dammarane-type triterpenes	[221]
	716A52	β-amyrin 28-oxidase	Oleanane-type triterpenes Oleanolic acid	[77]
	716A53	Protopanaxadiol 6-hydroxylase	Dammarane-type triterpenes	[222]
Papaver somniferum	80B3	N-methylcoclaurine 3′-hydroxylase	Benzoisoquinoline alkaloids Reticuline	[223]
	82N4	N-methylstylopine 14-hydroxylase	Protoberberine and benzoisoquinoline alkaloids	[224]
		N-methylcanadine 14-hydroxylase	Sanguinarine	
	82Y1	N-methylcanadine 1-hydroxylase	Phthalideisoquinoline alkaloids	[225]
		N-methylstylopine 1-hydroxylase	Noscapine	
	719A21	Canadine synthase	Phthalideisoquinoline alkaloids Noscapine	[226]
	719B1	Salutaridine synthase	Morphinan alkaloidsMorphine	[227]
Sesame indicum	81Q1	Dimerization of pinoresinol	Furofuran lignan Sesamin	[83]
Taxus brevifolia	725A1	Taxane 10β-hydroxylase	Diterpenoids Paclitaxel	[228]
	725A2	Taxane 13α-hydroxylase	Diterpenoids Paclitaxel	[229]
	725A3	Taxane 14β-hydroxylase	Diterpenoids Paclitaxel	[230]
	725A-like	Taxadiene 5a-hydroxylase	Diterpenoids Paclitaxel	[231]
	725A-like	Paclitaxel 2a-hydroxylase	Diterpenoids Paclitaxel	[232]
	725A-like	Taxadiene 7β-hydroxylase	Diterpenoids Paclitaxel	[233]

P450 reductases, including two in *A. thaliana,* three each in *O. sativa, P. tricocarpa* and others, have indicated that CPR proteins fall into distinct clusters with the CPR1 cluster restricted to dicots and the CPR2 cluster present in dicots and monocots [3, 89]. Within the same species, conservations among the CPR sequences are moderate with 63 % identity between the two in *Arabidopsis* and 72 % identity between the three in *Oryza*. Even more divergence exists among the cyt b5 sequences with 35–67 % identity between the five in *Arabidopsis* and substantially more among the many in *Oryza*. While the physiological roles of these many CPR and cyt b5 proteins are unclear, it has been repeatedly suggested that they interact with different subsets of ER-localized P450s. Evidence in support of this exists only in a recent study showing that the *Oryza* CPR2 enhances cinnamic acid 4-hydroxylase activity significantly better than either *Oryza* CPR1 or CPR3 [90]. Evidence in refutation of this exists in an older study showing that both *Arabidopsis* CPR proteins support cinnamic acid 4-hydroxylase activity [91].

7.2.7 Genomic Resources

In contrast to the large quantity of genome and transcriptome information available for model plants on a variety of websites (*A. thaliana* (http://arabidopsis.org), *O. sativa* (http://rice.plantbiology.msu.edu; http://rapdb.dna.affrc.go.jp) and others), genome information is not yet available for most medicinal plants. Conse-

quently, transcriptome sequencing efforts have been mounted in the recent years via large-scale efforts on multiple species (Phytometasyn, http://www.phytometasyn.com, [92]; Medicinal Plant Genomics Consortium, http://medicinalplantgenomics.msu.edu, [93]) as well as smaller-scale efforts on individual species (*C. roseus* [94, 95]; *G. uralensis* [96]; *P. somniferum* [97–99]; *P. ginseng* [100]; *T. cuspidata* [101, 102]; *T. mairei* [103]). Large-scale genome and transcriptome sequencing efforts are underway for many conifer species subject to insect and fungal infestations, including sitka spruce (*Picea sitchensis* [104]); white spruce (*Picea glauca* [105], http://www.smartforests.ca), Norway spruce (*Picea abies* [106], http://congenie.org); loblolly pine (*Pinus taeda* [107], http://www.pinegenome.org/pinerefseq), lodgepole pine (*Pinus contorta*) and jack pine (*Pinus banksiana* [108]).

Coupled with metabolite analyses of natural plant mutants and/or ecotypes deficient in particular compounds as well as engineered plant lines silenced for particular P450s, these genomic and transcriptomic resources are providing details on the exceptionally large number of P450 transcripts expressed in different plant species, those co-regulated in branch pathways and metabolic interactions between primary and specialized compounds in individual species.

7.2.8 Gene Clusters

With the increasing amount of genomic information available, it is becoming evident that some previously mentioned plant P450s are physically clustered and co-regulated with other genes (OMT, NMT, oxidoreductase, etc.) in their biochemical pathways. First evident in the *Z. mays* CYP71C cluster for hydroxamic acid synthesis [109], P450 clusters for specialized products have now been annotated for avenacin synthesis in *Avena* spp. [110], momilactone and phytocassane syntheses in *O. sativa* [23, 48, 49, 111], thalianol and marneral syntheses in *A. thaliana* [85, 112], noscapine synthesis in *P. somniferum* [113], and cyanogenic glucoside and triterpene syntheses in *L. japonicus* [78, 114]. Although the evolutionary origins of these gene clusters are not clear, it is worth noting that the pathway clusters already identified contain between one and six P450 genes with multiple P450s frequently, but not always, within the same subfamily. As summarized in Field and Osbourn [85] and Chu et al. [115], there are obvious advantages to maintaining P450 genes in close proximity to non-P450 genes in synthetic pathways, including the probability that they will be co-inherited and co-regulated despite being in independent transcription units.

7.2.9 Critical Structural Regions

7.2.9.1 Classical Monooxygenases

Coupled with structural predictions, natural and engineered variations in several of these plant P450s have identified critical residues in SRS and non-SRS regions that are reviewed in Rupasinghe and Schuler [116], Hlavica and Lehnerer [117] and Schuler and Rupasinghe [29]. Examples of natural side-chain SRS variations affecting P450 regiospecificity include the C6- versus C3-limonene hydroxylases of *Mentha spicata* (spearmint) CYP71D18 and *M. piperita* (peppermint) CYP71D15 sequences that have a single Phe363Ile switch in SRS5 dictating their respective activities [118]. Examples of synthetic site-directed SRS variations affecting substrate positionings and activities include the *Helianthus tuberosus* (jerusalem artichoke) CYP73A1 (4-cinnamic acid hydroxylase) that has Asn302, Ala306, and Ala307 in SRS4 (I-helix), Ile371 and Pro372 in SRS5 (loop between the K-helix and β1–4 strand), and Lys484 in SRS6 (β-turn at the end of β-sheet 4) dictating its reactivities [119, 120], *N. tabacum* CYP71D20 (5-epiaristolochene 1,3-dihydroxylase) that has Ser368 in SRS5 and Ile486 in SRS6 controlling its overall activity [121], *H. muticus* CYP71D55 (premnaspirodiene oxygenase) that has Val366 in SRS5 and Val480, Val482 and Ala484 in SRS6 (aligning with Ser482, Ile484 and Ile486 in CYP71D20) affecting catalytic site geometry [122], *Vicia sativa* (vetch) CYP94A2 (fatty acid

ω-hydroxylase) that has Phe494 in SRS6 affecting hydroxylation positions on short-chain fatty acids [123], *G. echinata* (licorice) CYP93C2 (2-hydroxyisoflavonone synthase) that has Ser310 in SRS4 (in place of the oxygen-activating Thr) and Leu371 and Lys375 in SRS5 controlling aryl migrations occurring in its substrate [82], *Gerbera hybrida* (gerber daisy) CYP75B15 (flavonoid 3′-hydroxylase) that has Thr487 in SRS6 controlling substrate positioning [124] and *A. annua* CYP71AV1 that has Ser479 in SRS6 controlling the second oxidation on amorpha 4,11-diene [125]. Evidence that these and other small changes in catalytic site residues can alter metabolic activities exist in several of these previously mentioned studies as well as in a recent study detailing adaptive changes in the CYP79F subfamily of *Boechera stricta* (close relative of *A. thaliana*) where a Gly134Leu changes in SRS1 and a Pro536Lys change five amino acids from the C-terminus allow for the synthesis of new glucosinolates [126].

Likely due to the restricted targeting of site-directed mutations to SRS regions in plant P450s, there are few examples of non-SRS variations affecting catalytic activities. Some that do exist are *Triticum aestivum* (wheat) CYP98A subfamily members that have an additional Cys52 at the N-terminus of their A-helices orienting ρ-coumaroyltyramine for its *meta*-hydroxylation [127].

7.2.9.2 Non-classical Monooxygenases

Compared to the classical endoplasmic reticulum-localized P450s that utilize molecular oxygen, the nonclassical *A. thaliana* and *Parthenium argentatum* (guayule) CYP74A proteins (allene oxide synthases) are chloroplast-localized, soluble and extremely unusual in using hydroperoxides as oxygen donors without the need for an electron transfer partner [128]. Contributing to these atypical properties, these P450s have an atypical insertion of nine residues upstream from their heme Cys ligand. Structure determinations on the *A. thaliana* and *P. argentatum* CYP74A proteins [129, 130] have indicated that this insertion reorganizes external surfaces potentially interacting with electron transfer partners and, in *A. thaliana* CYP74A1, repositions the I-helix kink so that Asn321 is over the heme and Ile328 replaces the catalytically important Thr. Even with these structural differences, other SRS residues remain important for allene oxide formation and their replacements convert one CYP74 subfamily protein into another. Site-directed replacement of Phe137 in SRS1 of *A. thaliana* CYP74A1 with Leu allows for 13-hydroperoxide cleavage (an activity characteristic of the CYP74B subfamily) rather than cyclization (an activity characteristic of the CYP74A subfamily) [129]. In other examples, replacement of Glu292 (in SRS4) and Val379 of *N. tabacum* CYP74D3 with Gly and Phe converts it from a divinyl ether synthase (an activity of the CYP74D subfamily) to a cyclizing allene oxide synthase [131].

In short summary, plant P450s are evolving at varying rates depending on their catalytic functions. With the new metabolic pathways evolving as ecological pressures dictate, the P450 gene counts in individual plant species have increased in manners allowing for the acquisition of new functions while maintaining critical catalytic functions. With activities defined for just a small fraction of all sequenced plant P450s, there is much to be learned about plant metabolic pathways from the biochemical and molecular analyses of individual monooxygenases.

7.3 Insect P450s

7.3.1 Gene Counts

Sequencings of a relatively small number of insect genomes (of more than 950,000 insect species) have identified substantially fewer P450 genes than in most plant species. Specifically, there are 87 in *Bombyx mori* (silkworm), 76–91 in *Drosophila* spp. (“fruit flies”), 105–180 in *Anopheles gambiae, Aedes aegypti,* and *Culex pipiens* (mosquitoes), 46 in *Apis mellifera* (honey bee), 106 in *Nasonia vitripennis* (jewel wasp), 134 in *Tribolium casteneum* (red flour beetle), 64 in *Acrythosiphon pisum* (pea aphid), 36 in *Pediculus humanus* (body louse), and 85 in *Dendroctonus ponderosae* (mountain pine beetle) [6, 234–240].

7.3.2 Gene Conservations and Divergences

Comparisons among the P450 sequences annotated in completed insect genomes, which are available in a number of recent reviews [241, 242], identify four clans including the CYP2, CYP3, CYP4, and mitochondrial groupings. Of these, the CYP2 clan contains 10 families, the CYP3 clan contains 30 families, the CYP4 clan contains 16 families, and the mitochondrial clan contains 11 families. Most of those conserved in insect species occur in the CYP2 and mitochondrial clans and include the CYP302A, CYP306A, CYP307A, CYP314A, CYP315A subfamilies in ecdysteroid synthesis [243], the CYP18A subfamily in ecdysteroid inactivation [244], and the CYP15 family in juvenile hormone synthesis [245]. Less conserved in insect species are those in the expanded CYP4 clan that contains many uncharacterized P450 families and the substantially more expanded CYP3 clan that contains numerous CYP6 and CYP9 family members associated with xenobiotic metabolism.

Within these last two clans whose gene numbers vary most among insects, repeated duplications within some subfamilies have given rise to "blooms" of P450s that often are species-specific and likely associated with host plant usage [246]. Examples here include: the expansion of the 19-member CYP4AB subfamily in *N. vitripennis* [238], 15-member CYP6AS subfamily in *A. mellifera* [235], 13-member CYP6BQ subfamily in *T. casteneum* [236], 12-member CYP6A subfamily in *D. melanogaster,* 9-member CYP9A subfamily in *Spodoptera frugiperda* (fall armyworm) [247], and the CYP6AB and CYP6AE subfamilies in *Amyelois transitella* (navel orangeworm) [248]. As in many of the plant genomes, many of these reiterated P450 subfamilies remain clustered within insect genomes [242].

7.3.3 P450s in Model and Nonmodel Insects

The current list of activities for insect P450s characterized via one of the heterologous expression systems mentioned above is presented in Table 7.4. As detailed, four of the five conserved subfamilies in 20-hydroxyecdysone (20-HE) synthesis (CYP302A, CYP306A, CYP314A, CYP315A) have been characterized from *D. melanogaster, B. mori,* and/or *A. gambiae* [243]. The remaining CYP307A1 subfamily, which has not yet been heterologously expressed, is nonetheless heavily implicated in ecdysteroid synthesis [243, 249]. Balancing these 20-HE synthetic activities, the conserved CYP18A subfamily has been shown to mediate 20-HE inactivation via its 26-hydroxylation [244]. Less conserved than members of these six ecdysteroid-metabolizing subfamilies, multiple CYP15 family members have been shown to code for epoxidations in juvenile hormone (JH) synthesis [245]. Categorized in different subfamilies, these vary in their substrate preferences depending on whether they have been obtained from lepidopteran species (e.g., CYP15C1 in *B. mori*) that first epoxidize and then methylate farnesoic acid or from other species (e.g., CYP15A1 in *Diploptera punctata* (pacific beetle cockroach)) that do these reactions in the reverse order [245]. Balancing these JH synthetic activities, other P450s inactivate JH via its 12-hydroxylation. Expected to be conserved among insects, it is surprising that only two P450s in different families (*D. melanogaster* CYP6A1, *D. punctata* CYP4C7) have been shown to mediate JH catabolism [250, 251]. Other activities expected to be conserved among insect species include those mediating fatty acid hydroxylations; here again, it is surprising that only one (*D. melanogaster* CYP6A8) has been shown to have any ability to oxygenate fatty acids [252].

Other biosynthetic P450s in insects have been sporadically identified as researchers have sought to delineate species-specific conversions involved in the production of insect defense toxins and pheromones. Recently characterized in *Zygaena filipendulae* (burnet moth) larvae, CYP332A3 and CYP405A2 are responsible for the synthesis of the cyanogenic glycosides linamarin and lotaustralin from valine and isoleucine, respectively [253]. Mediating multiple steps converting amino acids to cyanogenic glycosides,

Table 7.4 Functionally defined P450s in insects

P450	Species	Substrates	References
Insects			
4C7	*Diploptera punctata*	Farnesol, farnesal, farnesoic acid, methyl farnesoate, JHIII	[251]
6A1	*Musca domestica*	Farnesal, methyl farnesoate, JHI, JHIII, steroid hormones, cyclodienes, organophosphates, aldrin, heptachlor, diazinon, chlorfenapyr, pisatin	[272] [250] [273] [329] [316]
6A2	*Drosophila melanogaster*	DDT, aldrin, heptachlor, diazinon, aflatoxin B1, DMBA, Trp-P-2	[330] [315] [276]
6A8	*Drosophila melanogaster*	Lauric acid, aldrin	[252]
6B1,6B3	*Papilio polyxenes*	Furanocoumarins, furanochromones, flavone	[308] [259] [262] [263]
6B4,6B17,6B21	*Papilio glaucus*	Furanocoumarins	[259] [260]
6B33	*Papilio multicaudatus*	Furanocoumarins	[266] [267]
6B8	*Helicoverpa zea*	Xanthotoxin, flavone, α-naphthoflavone, chlorogenic acid, indole-3-carbinol, quercetin, rutin, cypermethrin, diazinon, aldrin	[261] [269]
6D1	*Musca domestica*	Pyrethroids, polycyclic aromatic hydrocarbons, methoxyresorufin	[331] [332] [280]
6G1	*Drosophila melanogaster*	DDT, imidacloprid, methoxychlor, *p*-nitroanisole	[277] [278] [279] [333]
6M2	*Anopheles gambiae*	Permethrin, deltamethrin, DDT	[299] [300]
6P3	*Anopheles gambiae*	Permethrin, deltamethrin	[334]
6P7	*Anopheles minimus*	Pyrethroids	[304]
6Z1	*Anopheles gambiae*	Furanocoumarins, furanochromones, methylenedioxy-phenyls, cypermethrin, DDT, carbaryl	[297] [302]
6Z2	*Anopheles gambiae*	α-naphthoflavone, resveratrol, piceatannol, xanthotoxin, carbaryl, 3-phenoxybenzoic alcohol, 3-phenoxybenzaldehyde	[297] [298] [301]
6Z8	*Aedes aegypti*	3-phenoxybenzoic alcohol, 3-phenoxybenzaldehyde, benzyloxyresorufin, ethoxyresorufin, α-naphthoflavone, resveratrol, diethylstilbesterol, pyriproxifen	[301]
6AA3	*Anopheles minimus*	Deltamethrin	[303] [304]
6AB3	*Depressaria pastinacella*	Imperatorin, myristicin	[264] [265] [268]
6AB11	*Amyelois transitella*	Imperatorin	[271]
6AS1,6AS3,6AS4,6AS10	*Apis mellifera*	Quercetin	[306]

Table 7.4 (continued)

P450	Species	Substrates	References
6AY1	*Nilaparvata lugens*	Imidacloprid	[335]
6BQ9	*Tribolium casteneum*	Deltamethrin, benzyloxyresorufin	[281]
6BQ23	*Meligethes aeneus*	Deltamethrin, *tau*-fluvalinate, 7-benzyloxymethoxy-4-trifluoromethyl coumarin, 7-benzyloxy-4-trifluoromethyl coumarin, 7-benzyloxymethoxyresorufin	[336]
6CM1	*Bemisia tabaci*	Imidacloprid, clothianidin, thiacloprid, pymetrozine, ethoxycoumarin, ethoxyresorufin, methoxyresorufin, benzyoxyresorufin	[282] [283] [284]
6CY3	*Myzus persicae*	Nicotine, imidacloprid, clothianidin	[274]
9A12,9A14	*Helicoverpa armigera*	*p*-nitroanisole, methoxyresorufin, esfenvalerate	[285]
9J24,9J26,9J28,9J32	*Aedes aegypti*	Permethrin, deltamethrin	[337]
9Q1,9Q2,9Q3	*Apis mellifera*	Quercitin, *tau*-fluvalinate, coumaphos, bifenthrin	[275]
9T1	*Ips confusus*	Myrcene	[255]
9T2 9T3	*Ips pini*	Myrcene, pinene, carene, limonene	[254] [255] [256]
12A1	*Musca domestica*	Aldrin, diazinon, heptachlor, azinphosmethyl, amitraz, steroids, 7-alkoxycoumarins	[338]
15A1	*Diploptera punctata*	Methyl farnesoate	[339]
15C1	*Bombyx mori*	Farnesoic acid	[340]
18A1	*Drosophila melanogaster*	20-hydroxyecdysone	[244]
302A1	*Drosophila melanogaster* *Bombyx mori* *Anopheles gambiae*	2,22-dideoxyecdysone	[341] [342] [343]
306A1	*Drosophila melanogaster* *Bombyx mori*	2,22,25-trideoxyecdysone	[344] [345]
314A1	*Drosophila melanogaster* *Anopheles gambiae*	Ecdysone	[346] [347] [343]
315A1	*Drosophila melanogaster*	2-deoxyecdysone	[341]
	Anopheles gambiae	2,22-dideoxyecdysone	[343]
321A1	*Helicoverpa zea*	Furanocoumarins, α-naphthoflavone, cypermethrin, diazinon, aldrin, aflatoxin B1	[312] [269] [270]
332A3	*Zygaena filipendulae*	Valine- and isoleucine-derived oximes	[253]
345E2	*Dendroctonus ponderosae*	Monoterpenes	[289]
405A2	*Zygaena filipendulae*	Valine, isoleucine	[253]
Mites			
392A16	*Tetranychus urticae*	Abamectin, luciferin, 7-ethoxy-4-trifluoromethyl coumarin, 7-ethoxycoumarin	[287]
392E10	*Tetranychus urticae*	Spirodiclofen, spiromesifen	[286]

DDT dichlorodiphenyltrichloroethane, *DMBA* 7,12-dimethylbenz(a)anthracene

these P450s have convergently evolved the same sorts of sequential conversions used in the synthesis of dhurrin, linamarin, and lotaustralin in cyanogenic plants (sorghum, cassava, lotus) [253]. Characterized in *Ips paraconfusus* (california fivespined ips) and *Ips pini* (pine engraver beetles), the CYP9T subfamily mediates both biosynthetic and detoxicative reactions in using the monoterpene myrcene present in conifer bark as the substrate for the production of aggregation pheromone [254–256], a mixture of ipsdienol, ipsenol, and other volatiles that recruit other beetles to damaged trees [257]. And, because they cannot rely solely on the presence of plant-derived myrcene, male bark beetles are also capable of synthesizing myrcene *de novo* and converting it into ipsdienol and ipsenol [257]. Biochemical characterizations of the species-specific differences between members of the CYP9T subfamily have shown that *I. paraconfusus* CYP9T1 utilizes myrcene to produce ipsdienol and ipsenol [255] and *I. pini* CYP9T2 and CYP9T3 (isolated from geographically distinct regions) utilize myrcene, pinene, carene, and limonene to produce ipsdienol and an array of other volatiles [254–256]. Another enzyme implicated in pheromone production is the *Dendroctonus ponderosae* (mountain pine beetle) CYP6CR1, which has been suggested to mediate the male-specific fatty acid epoxidation leading to production of the pheromone *exo*-brevicomin and another unidentified P450 has been suggested to mediate the female-specific hydroxylation of ingested α-pinene to the pheromone verbenol [258].

While the CYP15A and CYP15C subfamily members in 20-HE synthesis provide evidence that catalytic site differences can impact reaction orders in synthetic pathways, there are many more examples providing evidence that catalytic site divergences impact substrate preferences in detoxicative pathways. Originating in studies to understand the ecological bases for host plant ranges and shifts in nonmodel insects, catalytic site restrictions decreasing substrate range have been noted in the evolution of the CYP6B subfamily of *Papilio* spp. (swallowtails), *Helicoverpa zea* (corn earworm), and *Amyelois transitella* (navel orangeworm) and the CYP6AB subfamily of *Depressaria pastinacella* (parsnip webworm) and *A. transitella,* where duplications and divergences of subfamily members have allowed some specialist species to feed on a limited number of toxin-containing plant species and other generalist species to feed on a diverse array of toxin-containing plant species [259–271]. Contrasting with these, catalytic site accommodations enhancing substrate range have been noted in CYP6B8 and CYP321A1 of *Helicoverpa zea* (cotton bollworm) [269], CYP6A1 of *Musca domestica* (house fly) [250, 272, 273], CYP6CY3 of *Myzus persicae* (green peach aphid) [274], the CYP9Q subfamily of *A. mellifera* [275], and various subfamilies of mosquito vectors (discussed below), where divergences of individual P450s have allowed them to mediate the detoxification of plant compounds as well as insecticides. With no information available on their natural substrates, activities capable of catabolizing insecticides have been noted for CYP6A2 and CYP6G1 in *D. melanogaster* [276–279], CYP6D1 in *M. domestica* [280], CYP6BQ9 in *T. casteneum* [281], CYP6CM1 in *Bemisi tabaci* (white fly) [282–284], the CYP9A subfamily in *Helicoverpa armigera* (cotton bollworm) [285], *Nilaparvata lugens* (brown planthopper) and the CYP392 family in *Tetranychus urticae* (two-spotted spider mite) [286, 287]. In addition to these reactions detoxifying insecticides, several P450s have been shown to activate proinsecticides into toxic derivatives (e.g., chlorferuron via N-dealkylation, chlorfenapyrdiafenthiuron via S-oxidation) [288]. Characterized for its ability to catabolize natural compounds and not insecticides, CYP345E2 in *D. ponderosae* has been shown to mediate the clearance of monoterpene odorants [289]. Other insect oxygenations attributed to as-yet-uncharacterized P450s are reviewed in Feyereisen [242].

7.3.4 P450s in Vector Insects

Much of the research in insects vectoring human disease has centered on mosquito species, including *Anopheles* spp. (malaria vectors), *Ae. aegypti*

(dengue and yellow fever vectors), and *Culex* spp.(west nile vector), that are becoming increasingly resistant to insecticides. Microarray and transcriptome analyses in each of these species have identified varying sets of P450 transcripts in the CYP4, CYP6, CYP9, CYP12, CYP305, CYP307, CYP314, and CYP325 families constitutively overexpressed in different insecticide-resistant populations (compared to insecticide-susceptible populations) [242, 290, 291] and induced by fluoranthene, permethrin, glyphosate, and benzopyrene in *Ae. aegypti* or by permethrin in *C. quinquefasciatus* [292–296]. Narrowing the range of P450 candidates mediating the metabolism of different insecticide classes, subsequent expressions in insect cell systems have identified a range of insecticides catabolized by CYP6M2, CYP6P3, CYP6Z1, and CYP6Z2 in *A. gambiae* [297–302], CYP6P7 and CYP6AA3 in *A. minimus* [303, 304], CYP6Z8 and the CYP9J subfamily in *Ae. aegypti* [301]. Summarized in Table 7.4, these heterologous expressions have identified some, such as CYP6Z1, metabolizing many classes of insecticides (carbamates, type I and type II pyrethroids, DDT analogues), plant defense toxins (furanocoumarins, furanochromones), and natural and synthetic methylenedioxyphenyl (MDP) compounds (safrole, isosafole, myristicin, piperonyl butoxide) [297, 302], others, such as CYP6Z2 and CYP6Z8, metabolizing pyrethroid derivatives produced by carboxyesterases (3-phenoxybenzoic acid, 3-phenoxybezaldehyde) [301], and yet others, such as CYP6M2, mediating multiple modifications on deltamethrin [299]. Coupled with transcriptome data showing variable sets of P450s overexpressed in different insecticide-resistant strains and populations, it is becoming clear that the long-term outcome of exposure to insecticides is determined by the expression levels of multiple P450s and by the overlaps in their substrate profiles.

7.3.5 Critical Structural Regions

While these examples highlight the breadth of compounds metabolized by insect P450s, comparisons of the predicted catalytic sites have provided more significant information on the amino acid variations between nonselective and selective P450s. Recent reviews [291, 305] provide numerous examples of the variations affecting particular P450 activities with most examples drawn from comparisons of closely related subfamily members and not from natural variations in individual insect P450s. Not surprisingly, therefore, most of the highlighted variations map to residues in catalytic sites, substrate access channels, and proximal surfaces. Highlighting a few of the important differences between closely related subfamily members, instances of catalytic site differences include *A. gambiae* CYP6Z2, where protrusions of Arg210 (SRS2), Ile298 and Glu302 (both in SRS4) are predicted to restrict its substrate range compared to CYP6Z1 [297], *A. mellifera* CYP9Q2, where protrusion of Arg246 (SRS3) into its catalytic site is predicted to prevent quercetin metabolism compared to CYP9Q1 [275] and the *A. mellifera* CYP6AS subfamily, where side chains on residues 107 (SRS1) and 217 (SRS2) and the carbonyl backbone between residues 302 and 303 (SRS4) moderate quercetin metabolism [306]. Many other examples of catalytic site variations affecting activity exist in the *Papilio* and *Helicoverpa* CYP6B subfamily, where furanocoumarin metabolism rates are defined by the presence or absence of aromatic side chains in SRS1, SRS5, and SRS6 and other types of side chains in all six SRS regions [291]. Instances of substrate access channel differences affecting activity include *A. minimus* CYP6P8, where Arg114 (SRS1) and Arg216 (SRS2) are predicted to extend into the CYP6P8 substrate access channel and prevent pyrethroid access compared to the closely related CYP6P7 that metabolizes this insecticide quite efficiently [307].

Characteristic of the small number of natural and site-directed variants actually analyzed, some natural *P. polyxenes* CYP6B3 variants [263] and site-directed *P. polyxenes* CYP6B1 mutants [308–310] have identified particular SRS residues important in P450 folding, substrate turnover, and/or product exit. Adding to this collection of important residues, natural *D. pastinacella* CYP6AB3 variants have identified

proximal surface residues affecting catalytic efficiency with a single Val92Ala (B-helix on the proximal surface) switch substantially enhancing electron transfer from P450 reductase [265]. And, several site-directed *P. multicaudatus* CYP6B33 mutants have identified residue 32 (in the linker preceding the proline-rich hinge) as important for folding of this P450 in insect cell expression systems [267]. Not yet tested in site-directed mutants, other examples of potentially important residues likely exist in CYP6CM1 variants, where two changes in imidacloprid-resistant *B. tabaci* (His341Asn, Asn367Thr (numbered as in resistant compared to susceptible biotypes)) map to the proximal surface [282], CYP6A2 variants, where two changes in DDT-resistant *D. melanogaster* (Arg335Ser, Leu336Val) map to the proximal surface [276], CYP6B7 variants, where three changes in fenvalerate-resistant *H. armigera* (Val144Met, Glu256Lys, Cys319Tyr) map to D-helix, I-helix and G-H loop segments on the proximal surface [311] and CYP6Z1 variants where one change in permethrin- and DDT-resistant *A. gambiae* (Thr346Asn) maps to the proximal surface [297]. Summaries of these and other variations contributing to metabolism [305] show just how few amino acid differences in insect P450s have been evaluated in site-directed mutants.

7.3.6 Unusual Features

7.3.6.1 Reactivities and Residues

With characterized insect monooxygenases mediating more typical oxidations than those found in plants, there are fewer examples of insect P450s with unusual substrate reactivities and/or sequences in conserved regions. To date, only CYP6M2 of *A. gambiae* has been shown to mediate multiple modifications on deltamethrin that include hydroxylation and cleavage of its ether bond [299]. Likewise, only a handful of insect P450s contain significant changes in conserved motifs. Notable among these are the *A. gambiae* and *D. melanogaster* CYP307 proteins that contain an extremely unusual GGHSA(I/V) in place of (A/G)GX(D/E)*T*T (oxygen-activating Thr underlined) [79] as well as *H. zea* CYP321A1, *ptera litura* (oriental leafworm) CYP321B1, and *A. transitella* CYP321C1 and CYP321C3 [312, 313] (http://drnelson.uthsc.edu/cytochromeP450.html) that contain Pro in place of *T* and *D. melanogaster* CYP6G2 that contains Ser in place of *T* (http://p450.sophia.inra.fr/). Quite unusually, the CYP307 family proteins also have WXXXQ in place of the conserved WXXXR in the C-helix.

7.3.6.2 Inhibitor and Substrate Interactions

Unlike most other P450s, some insect P450s are not inhibited by natural MDP compounds, such as myristicin, or synthetic MDP compounds, such as piperonyl butoxide (PBO). Examples here include: *D. pastinacella* CYP6AB3 that metabolizes myristicin rather than allowing it to complex with heme [268] and *A. gambiae* CYP6Z1 that metabolizes PBO [302]. The atypical turnover of these compounds, which are generally presumed to inhibit P450 activities, have on occasion masked the role of P450s in particular metabolic processes.

Like CYP3A4 and other human P450s [314], some insect P450s (e.g., CYP6M2 in *A. gambiae*) and a mite P450 (e.g., CYP392E10 in *Tetranychus urticae* (two-spotted spider mite)) cooperatively bind their insecticide substrates [286, 300].

7.3.6.3 Electron Transfer Partners

Most sequenced insect genomes contain a single P450 reductase that shares 54–75 % identity with those in other insects [242]. Triple-transfections of insect cells with recombinant baculoviruses expressing P450, CPR and cyt b5 sequences have demonstrated that overexpression of these electron transfer partners can substantially enhance the low P450 activities typically obtained in insect cells transfected with recombinant virus expressing only P450 [262]. Single-transfections of insect cells with recombinant baculovirus expressing only P450 have shown that P450-containing cell lysates can be supplemented with purified CPR and cyt b5 proteins to enhance their P450 activities [315]. Apart from these appar-

ent translational limitations in heterologous expression systems, expressions of *M. domestica* CYP6A1 in *E. coli* cells and reconstitutions with purified electron transfer proteins have shown that the stability and activity of this monooxygenase are significantly enhanced by the presence of apo-cyt b5 [316]. Expressions and purifications of *A. minimus* CPR from *E. coli* cells under standard conditions have shown that this particular reductase is unstable and subject to significant flavin mononucleotide (FMN) loss because of atypical leucines in its FMN domain [317]. Conversion of these to the phenylalanines (Leu86Phe/Leu219Phe), which are found in other insect and vertebrate CPR proteins, and/or supplementation with FMN stabilize the *A. minimus* CPR and increase its activity with *A. minimus* CYP6AA3 [317, 318]; an additional Cys427Arg replacement in its predicted FAD-binding domain also increases activity [319]. Mechanistic studies have indicated that both the wildtype and Leu86Phe/Leu219Phe mutant forms of *A. minimus* CPR have a nonclassical two-site ping-pong mechanism for binding NADPH and cyt c [317, 318]. Expressions and purifications of *A. gambiae* CPR from *E. coli* cells have shown that this reductase is also subject to FMN and FAD loss and is inefficient in binding 2′, 5′ -ADP, which are all characteristics affecting its interaction and coupling efficiency with its associated P450s [320]. It is not yet clear what residues within the 2′, 5′ -ADP-binding site contribute to its poor performance and whether other insect CPR proteins have similar deficiencies.

7.3.7 Genomic Resources

Genome information is now accessible for multiple species of Drosophila (*D. melanogaster* and 20 others, http://flybase.org/), human and animal disease vectors (mosquitoes, sandflies, blackflies, tsetse fly, ticks, etc., https://www.vectorbase.org/), agronomic pests (hessian fly, house fly, red flour beetles, two-spotted spider mite, http://agripestbase.org/, http://www.spidermite.org/gapm/), arthropods (https://www.hgsc.bcm.edu/arthropod-sequencing, http://arthropodgenomes.org/wiki/Species_summary), Hymenoptera (bees, wasps, ants, http://hymenopteragenome.org/) and individual species such as *B. mori* (http://silkbase.ab.a.u-tokyo.ac.jp/cgi-bin/index.cgi)). For some of these species, P450-specific genomic information is available at http://p450.sophia.inra.fr/ and http://drnelson.uthsc.edu/cytochromeP450.html. With limited numbers of insect P450 activities characterized to date, many transcriptome sequencing projects are underway to provide comprehensive information on the range of P450s, GSTs, and UGTs constitutively or inducibly expressed in insecticide-resistant species. Recent transcriptome projects highlighting P450s include *Ae. aegypti* induced with atrazine (herbicide), fluoranthrene (polycyclic aryl hydrocarbon), propoxur, permethrin, and imidaclprid (insecticides) [295], *A. funestus* life stages [321], *A. gambiae* chemosensory appendages [322], *D. ponderosae* life stages, tissues and sexes [323–325], *Ips typographus* (spruce bark beetle) antennae [324], as well as comparative analyses in insecticide-susceptible, -resistant, and -selected strains (*Cimex lectularis* (bed bug), [326]; *A. gambiae,* [327, 328]).

In summary, these analyses have indicated that insects modulate expression of different subsets of P450s in their attempts to counter exposure to plant defense compounds and environmental xenobiotics. Current information suggests that, even within a single insect species, the array of P450s constitutively or inducibly expressed in response to selection pressure is not constant, with multiple subsets mediating the acquisition of toxin resistance in different populations and laboratory strains.

7.4 Fungal Pathogens

7.4.1 Gene Counts

Like plants and insects, fungi that interact with these species contain numerous P450s with roles in the production of primary and specialized metabolites as well as the detoxification and ca-

tabolism of natural compounds [348]. Genome sequencings have shown the fungal P450s to be extremely diverse and, sometimes, even more numerous than in some plants [349]. Characterizations of genomes and transcriptomes in multiple phytopathic fungi have identified 167 P450 genes in *A. flavus* (pathogen on corn, nuts), 131–136 in *Botrytis cinerea* (pathogen on grape) strains, 155 in *Aspergillus oryzae* (nonpathogen associated with soy fermentation) strains [349–351] (http://drnelson.uthsc.edu/, http://p450.riceblast.snu.ac.kr/), and at least 54 in *Grosmannia clavigera* (pathogen on lodgepole pines and other conifers) [352]. Genomes of basidiomycetes that infest other plants and degrade plant materials have exceptionally large collections of P450 genes with 149 in *Phanerochaete chrysosporium* (white-rot fungus), 353 in *Postia placenta* (brown-rot fungus), and 307 in *Moniliophthora perniciosa* (cocoa tree pathogen) [349, 353–355]. The presence of tandem arrays of P450 genes in these basidiomycetes suggest that they have been recently duplicated to allow for adaptation to the various functions associated with degradation of wood. Expansions of 11 P450 families in the basidiomycetes have yielded large families that are quite versatile and capable of accepting broad groups of substrates [355].

Substantially fewer characterizations exist for the genomes and transcriptomes of entomopathogenic fungi. Those completed to date have identified 83 and 123 P450 genes in *Beauveria bassiana* and *Metarhizium robertsii* (broad-range insect pathogens) that degrade cuticular layers in many insect species [356, 357] and 11 expressed P450 transcripts in *Ascosphaera apis* that exclusively infects honey bee larvae and causes chalkbrood disease [358].

7.4.2 Gene Conservations and Divergences

Found in most fungi, the CYP51 and CYP61 families, which have extremely small numbers of genes, mediate sterol 14α-demethylations and Δ22-desaturations, respectively, and the CYP52 family, which contains more genes, mediates hydroxylations of n-alkanes and fatty acids [348, 349]. Present in more limited fungal groups, the CYP53 family distributed in ascomycetes and basidiomycetes catalyzes hydroxylations of benzoic acid and its derivatives, which are plant phenylpropanoids, and the CYP505 family distributed in filamentous fungi catalyzes fatty acid hydroxylations. In significantly more limited groups of fungi, specialized pathways for the synthesis of aflatoxins in *A. flavus* and *A. parasiticus* are mediated by a cluster of CYP58, CYP59, CYP60, CYP62, and CYP64 genes [359], trichotecenes in *Fusarum* spp. are mediated by CYP58, CYP65, CYP68, CYP526 genes [360], fumonisins in *F. verticillioides* (maize pathogen) are mediated by CYP505 genes [361], and GAsin *F. fujikuroi* (rice pathogen) are mediated by CYP68, CYP69, and CYP503 genes [362]. Several of the P450 clusters involved in synthesis of these toxic metabolites also contain adjacent pathway genes needed for construction of the chemical backbone (e.g., polyketide synthase), export of the toxic metabolites (e.g., transporters), and transcriptional regulators. As a consequence, characterization of these pathways has been easier than in some of the plant species previously mentioned. Additionally, a P450 gene cluster in *Botrytis cinerea* has been shown to mediate the synthesis of ABA [363, 364].

In addition to these synthetic functions, several fungal P450s also have detoxicative functions toward plant compounds. Those characterized to date include the CYP57A subfamily members in *Fusarium, Nectria,* and *Neocosmospora* spp. that inactivate the isoflavonoid derivative pisatin and allow for infestation of pea plants [365], CYP57B3 in *A. oryzae* that hydroxylates genistein [366], CYP53D2 in *P. placenta* that O-demethylates methoxystilbene derivatives [353], and several CYP512 and CYP5150 family members that metabolize dehydroabietic acid [353]. Crossing the boundary between synthetic and detoxificative functions, CYP58 in *P. chrysosporium* has been predicted to participate both in synthesis of trichothecene (a fungal mycotoxin) and inactivation of benzoic acid (a plant phenylpropanoid)

[367]. Other detoxicative functions, such as CYP52X1 in the entomopathogenic fungus *B. bassiana,* are known to facilitate pathogenesis by oxidizing the long-chain fatty acids present in the protective layers of insect cuticles [368].

Residues important in some of these functionally characterized fungal P450s have been recently reviewed in Hlavica [369] (2013). With few studies detailing the P450s in plant and insect pathogens, most examples highlighted as important for catalytic activities are those in the common CYP51 and CYP61 families proteins present in many fungi, including *S. cerevisiae, Candida albicans* (pathogen in humans), and *Mycosphaerella graminicola* (pathogen on wheat).

7.4.3 Genomic Resources

Fungal P450 gene databases including more than 213 species exist at http://drnelson.uthsc.edu/ [6] (Nelson 2009) and http://p450.riceblast.snu.ac.kr/ [349, 370]. Limited information on the inducibilities of these is available from P450-specific oligoarrays in *P. chrysosporium* [354], the white-rot fungus that completely breaks down lignin, cellulose and hemicellulose, and from whole-genome microarrays in *P. chrysosporium* and *P. placenta* [371], the brown-rot fungus that does not completely break down lignin. These studies have shown that in lignin-degrading *P. chrysosporium,* CYP505D, CYP5037A, and CYP5141D subfamily members are highly induced by ligninolytic conditions and that multiple CYP63 family members are induced by alkanes [354]. Contrasting with these, only two P450s (CYP53 (benzoate hydroxylase), and CYP503 (undefined function)) are induced in lignin-nondegrading *P. placenta* by cellulose. Transcriptomic studies have also identified multiple P450 subfamilies induced and repressed by plant terpenes and triglycerides in *G. clavigera,* the conifer-invading fungus that is highly adapted to plant terpenoids [352]; notable among these is CYP65BJ1 in a biosynthetic cluster that produces aromatic polyketides. Clearly, much remains to be done in characterizing fungal P450s associated with pathogenesis in plants and insects.

7.5 Future Prospects

Clearly, plant and insect P450s abound. Our current understanding of their biochemistries has progressed as individual monooxygenases have been expressed in one or more of the available heterologous protein production systems and as natural variants and site-directed mutants have been characterized. Coupled with the growing body of P450 transcriptomic information available for a diverse array of plant species, there is much potential for the modification of metabolite profiles in transgenic plants and for the production of plant medicinals in microbial systems. Coupled with the transcriptomic information available for a sparser set of insect species, there is much potential for the identification of monooxygenases involved in the detoxification of insecticides and plant compounds and for their inhibition. Future studies in many of the species currently being explored will undoubtedly expand on the P450 examples cited in this chapter and begin to describe synthetic and detoxicative functions not yet characterized. Building on the sets of molecular, genetic, biochemical, and computational tools now available for manipulation of P450s in several model species, much remains to be done to fully understand the roles of individual P450s in normal growth and development as well as adaptation to new environments (plants) and new hosts (insects).

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P450 Biotechnology

8

Marco Girhard, Patrick J. Bakkes, Osama Mahmoud and Vlada B. Urlacher

8.1 Introduction

8.1.1 P450 Biotechnology: Application Potential

Oxyfunctionalization of nonactivated C–H bonds is one of the major challenges in chemistry. Nevertheless, this reaction type is crucial for the initial activation of simple starting molecules. Oxidation processes based on expensive and complex chemical catalysts, which have to be synthesized first—often via multistep processes—require harsh reaction conditions and are often not very effective. Although recent progress towards selective chemical hydroxylation of nonactivated C–H bonds has been made [1–3], a major disadvantage of most existing chemical catalysts still is their lack in selectivity [4]. In contrast, oxygenations of relatively cheap precursor molecules catalyzed by cytochrome P450 monooxygenases (P450 or CYP) in one step are often highly regioselective and stereoselective leading to high-value compounds that are difficult or even impossible to synthesize via traditional chemical routes [5]. In addition, P450s operate under mild reaction conditions utilizing molecular oxygen, which is abundant, environmentally friendly, and inexpensive. These characteristics make P450s potential biocatalysts for synthetic applications.

The history of P450s dates back to more than 50 years, when Klingenberg and Garfinkel independently discovered a carbon monoxide-binding pigment with a unique absorption maximum at 450 nm in rat and pig liver microsomes [6, 7]. Since their discovery, P450s have drawn the attention of chemists, biochemists and biotechnologists. During the last 20 years, P450s have gained interest not only from the viewpoint of advancing fundamental understanding but also from an industrial perspective. Their applications in the synthesis of oxyfunctionalized building blocks closely linked with the retrieval of new important compounds in demand (such as specialty chemicals and pharmaceutical synthons) are of immense importance. Moreover, P450s have a great potential for the development of biosensors, as well as in bioremediation.

Selective biocatalytic oxyfunctionalization of nonactivated hydrocarbons is considered as "potentially the most useful of all biotransformations" [8]. Cytochrome P450 enzymes contain heme b as prosthetic group that enables not only the activation of molecular oxygen (which is also possible by using flavin-containing enzymes) but also the oxidation of kinetically inert nonactivated C–H bonds. For instance, bond dissociation energies for *n*-alkanes lie within the range of 95–105 kcal mol^{-1}. Although no native

V. B. Urlacher (✉) · M. Girhard · P. J. Bakkes · O. Mahmoud
Institute of Biochemistry,
Heinrich Heine University Düsseldorf,
Universitätsstraße 1, 40225 Düsseldorf, Germany
e-mail: vlada.urlacher@hhu.de

M. Girhard
e-mail: marco.girhard@hhu.de

P. J. Bakkes
e-mail: patrick.bakkes@hhu.de

O. Mahmoud
e-mail: osama.mahmoud@hhu.de

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(wildtype) P450s with activity towards the most inert short chain length gaseous *n*-alkanes, such as methane, propane, or butane, have been identified so far, a number of P450s were discovered in nature with native activity towards pentane and longer alkanes.

Most P450s catalyze the reduction of molecular oxygen; one atom of molecular oxygen is introduced into the substrate molecule, whereas the second one is protonated to water (Eq. 8.1):

$$\begin{aligned} &\mathrm{NAD(P)H + RH + O_2 + H^+} \\ &\rightarrow \mathrm{R-OH + NAD(P)^+ + H_2O} \end{aligned} \quad (8.1)$$

The most typical reaction observed for P450s is hydrocarbon hydroxylation. In addition, P450s catalyze epoxidation of C=C double bonds and aromatic hydroxylation. After initial hydroxylation, subsequent reactions like alcohol oxidation; N-, O-, S-dealkylation; C–C bond cleavage; and others can occur, leading to a broad variability of potential reaction pathways [9]. Moreover, P450s do not only oxidize C atoms but also N and S atoms [10–12]. Some P450s are able to catalyze oxidative phenol coupling, a reaction that is usually carried out by peroxidases or laccases. For instance, three independent P450s with oxidative phenol coupling activities are involved in the synthesis of vancomycin-type antibiotics in the bacterium *Amycolatopsis balhimycina* [13]. Dimerization of thiophene S-oxide via a Diels–Alder reaction is catalyzed by CYP2C19 and CYP2D6 [14]. Baeyer–Villiger-type oxidations can also be catalyzed by some P450s [15]. The repertoire of P450 enzymes includes many other "unusual" reactions, such as oxidative deamination, oxidative dehalogenation, desaturation, isomerization, dehydrogenation, dehydration, reductive dehalogenation, epoxide reduction, and rearrangement reactions, such as ring formation and oxidative aryl migration [16–18]. The number of reported P450-catalyzed reactions is permanently increasing and numerous comprehensive reviews on this topic are currently available [17, 19–21]. A summary of the most common P450 reactions is given in the review by Sono et al. [22], where 21 different reaction types have been summarized, whereas an update of unusual reactions was recently provided by Guengerich and Munro [23]. Among the recently described unusual reactions catalyzed by P450s are nitration of tryptophan [24], cyclopropanation via carbene transfer [25], and intramolecular C–H amination [26].

P450s accept an extremely broad spectrum of organic substrate molecules, including fatty acids; alkanes; alkenes; mono-, di-, sesqui- and triterpenes (e.g., steroids); polyaromatic hydrocarbons; macrolides; heteroaromatic compounds; amino acids; and many others. Of course, there is no single P450 capable of accepting all these substrates. It is, however, relatively common for a certain P450 to metabolize multiple substrates [27]. Moreover, some P450s are reported to mediate multiple sequential modifications on a single substrate, which is particularly attractive when complex multistep biotechnological processes should be established.

The vast majority of P450 substrates are hydrophobic compounds with low solubility in water. The substrates are stabilized in the P450 binding pocket mainly via hydrophobic forces and van der Waals' forces and partially by electrostatic or π–π interactions. From the broad substrate spectrum on the one hand and a general preference for hydrophobic compounds on the other hand, it might be expected that P450 enzymes catalyze reactions with low stereoselectivity [28]. Contrary to this expectation, many P450s exhibit a high enantioselectivity towards racemic substrates or catalyze stereoselective introductions of oxygen into prochiral molecules.

In summary, cytochrome P450 monooxygenases have a number of advantages for biocatalysis:

1. P450s operate—like other enzymes—under ambient conditions.
2. P450s have been studied in enormous detail due to their involvement in a plethora of crucial cellular processes.
3. P450s are able to catalyze numerous different reaction types and can oxidize a wide range of molecules. Many of these compounds occur in nature and can be important precursors. Thereby, P450s often exhibit high regio-, chemo-, and/or stereoselectivity. In addition, enzyme engineering can be applied to further

Table 8.1 Challenges and limitations for biotechnological application of P450s

Challenge	Explanation or cause	Possible solution(s)
Low activity	Natural role of P450s	Protein engineering
	Complexity of catalysis	Fusions between monooxygenase and electron transfer proteins
Uncoupling	Poor fit of substrate to active site	Protein engineering
	Mismatch between redox partners	Redox chain optimization
Overoxidation	Product is also a substrate	In situ product removal
Cofactor depletion	Capacity of cell metabolism becomes limiting at higher oxygenase expression levels and/or activities	Coexpression of suitable enzymes for cofactor regeneration
Limited substrate uptake	Hydrophobic compounds disrupt cell membranes	Reduction of aqueous phase concentrations (e.g., by adsorption of substrates to a solid-phase or in situ product removal)
Substrate or product toxicity	General toxicity of polar compounds	Alternative hosts with altered uptake profiles
Product degradation		Coexpression of recombinant uptake systems
Limited oxygen transfer rates	Competition with endogenous respiration	Addition of oxygen
	Low $k_L a$ of standard bioreactors	Increased oxygen pressure
Low substrate solubility	Substrates are often hydrophobic and/or poorly soluble in water	Application of two-liquid phase systems (e.g., dissolving substrate in an inert organic solvent)
		Addition of cyclodextrins
		Addition of cosolvents (e.g., ethanol or dimethylsulfoxide)

improve the capabilities of P450s for biotechnological purposes.

4. P450s can be produced by fed-batch fermentation for production at large scale. Considerable progress has been made during the last decade concerning the recombinant expression of P450s in the well-established hosts *Escherichia coli, Pseudomonas putida,* and the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris,* which facilitates the use of P450s as industrial catalysts [29–33].
5. The number of identified P450s is enormous and constantly increasing due to microbial screenings and available information on sequenced genomes. The collection of P450s in (recombinant) libraries allows high-throughput screenings, as well as functional characterization of new members of the P450 family and offers a route to diverse building blocks.

8.1.2 P450 for Biotechnological Applications: Limitations

Despite their high potential, the application of P450 reactions at industrial scale has been hampered by several widely recognized bottlenecks (Table 8.1) [34].

1. In comparison with other enzyme classes (e.g., hydrolases), monooxygenases generally display low turnover numbers. This observation can be explained by their natural physiological roles and by the complexity of P450 catalysis, as well as by the high bond dissociation energies of P450 substrates. Such low activities might be sufficient for establishing P450-based biosensors, but mostly hamper applications in biocatalytic processes in industry.
2. For their activity, P450s require the consecutive delivery of two electrons to the heme. Nearly all P450s rely on the expensive pyridine nucleotide cofactors nicotinamide adenine dinucleotide phosphate (NADPH) or nicotinamide adenine dinucleotide (NADH), which makes large-scale applications of P450s not feasible if the cofactor has to be added in stoichiometric amounts.
3. Most P450 systems require complex multiprotein electron transfer chains. The search for suitable redox proteins that can efficiently deliver the electrons to the heme or even con-

struct man-made functional redox modules still remains a challenge. This is especially relevant for bacterial P450s since often many different candidates for electron transfer are present in a microbial cell [35]. The contribution of redox partners to the overall activity of P450s is often underestimated [36, 37]. Protein–protein interactions and efficient electron transfer between the redox partner proteins are essential reaction steps that have to be investigated on a case-by-case basis and adapted to allow for efficient oxygen activation and product formation.

4. Uncoupling between NAD(P)H oxidation and product formation may occur during the P450 reaction cycle, or, between the redox partners, which in turn leads to the formation of reactive oxygen species [38]. Moreover, upon consumption of two electrons, water can be produced without concomitant substrate hydroxylation [39–41]. In those cases, the cofactor NAD(P)H is consumed, but the formation of hydroxylated products is low. In addition, the reactive oxygen species may lead to instability and degradation of the heme cofactor and apoprotein. Uncoupling therefore represents another limitation in P450 biocatalysis.
5. Industrial applications of P450s have so far been restricted to whole-cell systems, which mostly solve the problem of cofactor delivery and regeneration. In such instances, however, physiological effects such as limited substrate uptake and reduced efflux of products out of cells, substrate or product toxicity, product degradation, as well as elaborate downstream processing are additional limiting factors that must be taken into account and often require optimization [34].

8.1.3 Outline

This book chapter focuses on recent advances in the application of cytochrome P450 monooxygenases in biotechnology. First, we will describe the exploitation of the natural pool of P450s for selective oxidation activities (Sect. 8.2). The engineering of P450s for higher activity, altered selectivity, and increased protein stability, as well as improved electron transfer between P450s and redox partners will be discussed in Sect. 8.3. A much-applied approach to optimize the electron flow is the design of fusion proteins, which is described in Sect. 8.4. Considerable progress has been made to overcome the need for stoichiometric amounts of NAD(P)H, either by developing effective cofactor regeneration systems or by designing new strategies for simplified transfer of reducing power; in Sect. 8.5, some of these strategies will be discussed. In Sect. 8.6, several examples of successful whole-cell biocatalysis exploiting P450s will be discussed, with special focus on microbial de novo synthesis of plant secondary metabolites and the generation of transgenic plants (Sect. 8.7).

Clearly, it is a daunting task to discuss all aspects of the rapidly developing field of P450 biotechnology and to review all recent publications. One should keep in mind that in 2013 alone, more than 2400 manuscripts (original papers, reviews, monographs in books) were published on P450s according to a literature search in the "Web of Science" database (apps.webofknowledge.com; 2014/03/27). Therefore, we will focus from a more academic point of view on several basic aspects and recent advances in P450 biotechnology.

8.2 New P450 Activities Found by Genome Mining and Microbial Screening

8.2.1 Natural P450 Pool for Selective Oxidations

P450s are ubiquitous in nature [42]. It is therefore obvious that genome mining represents an important tool that has already proved highly rewarding for the discovery of novel oxidation activities [43].

In "earlier day", the identification of new P450s was limited to classical in vivo screening of microbial strains, e.g., maintained in culture collections or identified by enrichment of cultures from natural sources. Even today, such traditional methods are still eligible and get con-

stantly improved [44]. However, often, considerable efforts are required for the setup and maintenance of such culture collections. Moreover, a major hurdle is that only a small percentage (0.01–1 %) of cells visible under the microscope will form colonies on a petri dish under laboratory conditions, leaving the remaining majority "uncultured" [45].

Advancing technologies have opened up new perspectives leading to the development of alternative screening strategies that aim to overcome the hurdles of traditional microbial screenings. One example is the screening of metagenome libraries of nonculturable microorganisms [46]. Probably one of the most promising strategies is the in silico screening of annotated P450 sequences from various sources that are available in online databases. The number of these sequences is rapidly increasing due to a vast number of genome sequencing projects. While during the first 40 years of P450 research between 1958 (when the first P450s were discovered [6, 7]) and 1998 less than 1000 P450 sequences were identified [47], their number approached 4000 in 2004 [48], 18,000 in 2011 [49], and crossed 21,000 in 2013 [42].

Several online databases allow genome mining via in silico screening for novel P450 enzymes. The "official" P450 database (also known as "the cytochrome P450 homepage") is maintained by David Nelson (http://drnelson.uthsc.edu/CytochromeP450.html; University of Tennessee; 2014/03/20) [50]. This database provides a classification of 21,000 P450 genes, inter alia including bacteria with 1254 genes, fungi with 5729 genes, plants with 7446 genes, insects with 3452 genes, and mammals with 1056 genes (status as of August 2013).

Another well-organized and structured database is represented by the "*Cy*tochrome *P450 E*ngineering *D*atabase" (CYPED; http://www.cyped.uni-stuttgart.de; Universität Stuttgart; 2014/03/20) [51, 52]. CYPED includes more than 16,000 sequences of P450s. In addition, information on 741 structures of P450s is integrated into this database to facilitate protein engineering.

Some more specialized databases for individual groups of P450 enzymes also exist. Examples are the "Fungal Cytochrome P450 Database" hosted in Korea and listing more than 8700 fungal P450 sequences from 113 species (http://P450.riceblast.snu.ac.kr; Seoul National University: 2014/03/20) [53], or the "SuperCYP" database that contains 1170 drugs, 2785 cytochrome P450-drug interactions, and 1200 P450 alleles (http://bioinformatics.charite.de/supercyp; Charité-University Medicine Berlin; 2014/03/20) [54].

The speed at which new P450 sequences are identified makes it increasingly difficult to keep up with the characterization of their (biochemical) properties. So far, only a limited number of annotated P450 sequences have been cloned and only few P450 enzymes have been functionally expressed and characterized. Nevertheless, reports on the biotechnological exploitation of naturally occurring and highly selective oxidations by P450 enzymes are accumulating and have recently been reviewed by our group [55]. Several examples of such reactions will be presented within this section.

8.2.2 Selective Oxidations of Alkanes and Fatty Acids

Alkanes and fatty acids represent interesting targets for biotechnological application of P450s. Hydroxylated alkanes are important synthons and precursor compounds for the synthesis of pharmaceuticals, agrochemicals, and liquid crystals [56]. Hydroxy fatty acids are widely used in the food and cosmetic industries. They serve as starting materials for the synthesis of polymers and as additives for the manufacture of lubricants, emulsifiers, and stabilizers. Furthermore, they have antibiotic, anti-inflammatory, and anticancer activities and therefore can be applied for medicinal uses [57]. It is therefore not surprising that P450-catalyzed hydroxylations of alkanes and fatty acids have been intensively studied. However, although these substrates are accepted by numerous P450s, the regioselectivities of the catalyzed hydroxylations are often insufficient, resulting in mixtures of hydroxylated products.

Only a few examples of naturally occurring highly regioselective P450s have been reported, mostly belonging to the bacterial CYP153 family, which has been described in detail in several reviews [58, 59]. A number of new CYP153A genes were isolated from different sources and applied in the form of recombinant *E. coli* whole-cell biocatalysts for biotransformations of *n*-alkanes and cylohexane. Up to 500 μg mL^{-1} of 1-hexanol or 1-octanol and 450 μg mL^{-1} of cyclohexanol could be produced with high regioselectivity [60]. Another study investigated several members of the CYP153A and CYP153D subfamilies catalyzing the oxidation of *n*-hexane, *n*-octane, and *n*-decane. Herein, >95% regioselectivity for terminal hydroxylation was observed with in vitro turnover rates reaching up to 58 min^{-1} for CYP153A6 with *n*-octane as substrate [61]. Utilization of CYP153A6 for the production of 1-octanol with recombinant *E. coli* allowed the production of 8.7 g L^{-1} 1-octanol within 24 h [62].

Other enzymes of the CYP153A subfamily show even higher regioselectivities: CYP153A16 from *Mycobacterium marinum* and CYP153A *P.* sp. from *Polaromonas* sp. exhibited 100% regioselectivity for terminal oxidation of *n*-pentane and *n*-hexane yielding the respective primary alcohols. In addition, CYP153A16 displayed 96% ω-regioselectivity for production of 1,8-octanediol from 1-octanol [63]. The potential of CYP153A16 and CYP153A *M. aq.* from *Marinobacter aquaeolei* for ω-hydroxylation of several saturated fatty acids was also investigated [64]. Both enzymes displayed 100% ω-regioselectivity with decanoic acid. Moreover, CYP153A *M. aq.* combined high ω-regioselectivity towards 9-monounsaturated fatty acids (90–100% depending on substrate) with moderate-to-high conversions (34–93%) [64].

Our group has recently identified CYP154A8 from *Nocardia farcinica* that catalyzes the stereo- and regioselective hydroxylation of C7–C9 *n*-alkanes. In a biphasic reaction system, the regioselectivity for the C2-position was more than 90% with total turnover numbers of up to 4400. The enzyme showed strict *S*-selectivity for all tested substrates, with enantiomeric excess (*ee*) of up to 91% [65].

P450 enzymes that are described in the context of regioselective fatty acid oxidation originate from the yeast CYP52 family. Well-studied members of the CYP52 family are the enzymes of the alkane and fatty acid-metabolizing yeasts *Candida maltosa, C. tropicalis,* and *Yarrowia lipolytica* [66–68]. In all these strains, a number of CYP52 genes were identified and investigated several years ago. One more recent example is CYP52A21 from *Candida albicans* demonstrating high regioselectivity for ω-hydroxylation of dodecanoic acid [69]. CYP52 family members CYP52E3, CYP52M1, and CYP52N1 from *Candida bombicola* have been suggested to catalyze terminal hydroxylations of fatty acids as well [70]. After heterologous expression of these enzymes in *Saccharomyces cerevisiae,* the functions of the recombinant proteins were analyzed with a variety of alkane and fatty acid substrates using either microsomal fractions or whole-cell systems [71]. While CYP52M1 was found to hydroxylate C16–C20 saturated and unsaturated fatty acids at their ω- and ω-1 positions, CYP52N1 oxidized C14–C20 saturated and unsaturated fatty acids exclusively at the ω-position. Minor ω-hydroxylation activities were also shown for CYP52E3 [71].

In addition to CYP52 enzymes, P450s belonging to the CYP4 family are also linked to ω-hydroxylation of fatty acids. In mammals, six CYP4 subfamilies have been identified. Three subfamilies show a preference in the metabolism of short (C7–C10; CYP4B subfamily), medium (C10–C16; CYP4A family), or long (C16–C26; CYP4F subfamily) saturated, unsaturated, and branched chain fatty acids [72]. While most reports on CYP4 enzymes have a medical background and focus on their involvement in genetic disorders and diseases [73, 74], the biotechnological potential of this family has not been explored so far. This is probably due to the fact that the handling of eukaryotic P450s is generally more difficult. Only a few enzymes have been heterologously expressed and characterized. Examples include human CYP4V2 [75] and rabbit CYP4B1 [76].

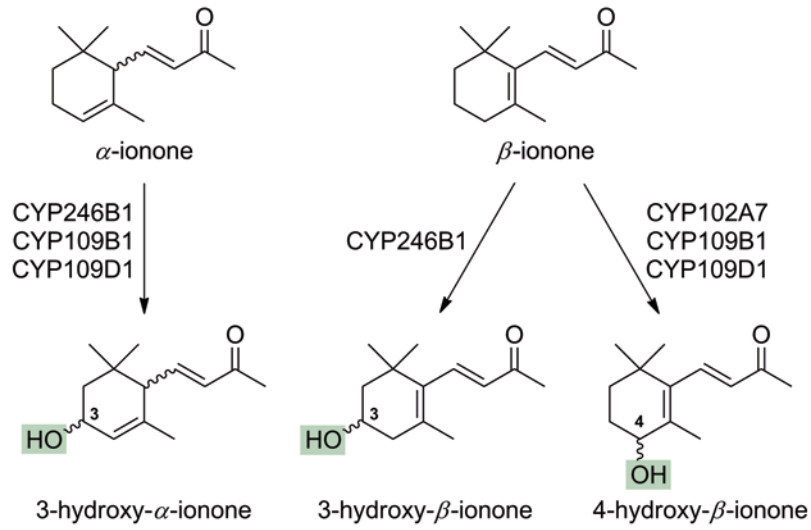

Fig. 8.1 Regioselective hydroxylations of α- and β-ionone by P450s

8.2.3 Selective Oxidations of Terpenes

P450-catalyzed regio- and enantioselective oxyfunctionalization of terpenes has evolved into an important research field [77, 78]. The importance of P450 oxidations is illustrated by the fact that more than 95% of the 60,000 known terpenoids are oxygenated. Oxygenated terpenoids are often high-priced and sought-after compounds for the food, fragrance, and pharmaceutical industries. Within this field, a driving force for novel biotechnological solutions is the fact that consumers show a strong preference for "natural" products. While according to the US and European food legislations, flavors that occur in nature but are produced by chemical synthesis must be called "nature-identical"; flavor substances originating from physical processes (extraction from natural sources), or from enzymatic or microbial processes that involve precursors isolated from nature are allowed to be labeled as "natural" [79].

Two examples, where the full potential of naturally occurring regioselective P450s is exploited, are the biotechnological production of artemisinic acid (a precursor of the antimalarial drug artemisinin) by CYP71AV1 from *Artemisia annua* [80] as well as production of 8-hydroxycadinene (a precursor for synthesis of the dimeric sesquiterpenoid gossypol) by CYP706B1 from *Gossypium arboretum* [81]. These (and other) examples will be described in detail in Sect. 8.7.

While it is less surprising that plant P450s are involved in oxidations of secondary plant metabolites, it seems rather unusual that also bacterial P450s are reported that are capable of regioselective oxidations of typical plant terpenes. Two terpenes of commercial interest are the regioisomeric α- and β-ionones, whose hydroxylated products are utilized as scents and building blocks for the synthesis of carotenoids and abscisic acid [82, 83]. Most P450s for which regioselective ionone oxidations have been described are of bacterial origin. Examples include CYP102A7 from *Bacillus licheniformis* [84] and CYP109B1 from *Bacillus subtilis* [85], as well as CYP109D1 [86] and CYP264B1 [87]—both from *Sorangium cellulosum* So ce56. The position of the carbon atom that is hydroxylated depends on the enzyme–substrate combination (Fig. 8.1): While α-ionone is not accepted by CYP102A7, β-ionone is exclusively oxidized to 4-hydroxy-β-ionone by this enzyme [84]. In contrast, CYP264B1 accepts both α- and β-ionone as substrates, but hydroxylation occurs exclusively at the C3 position [87]. CYP109B1 and CYP109D1 oxidize α- and β-ionone with 100% regioselectivity at the allylic carbon atom leading to the products 3-hydroxy-α-ionone and 4-hydroxy-β-ionone [85, 86].

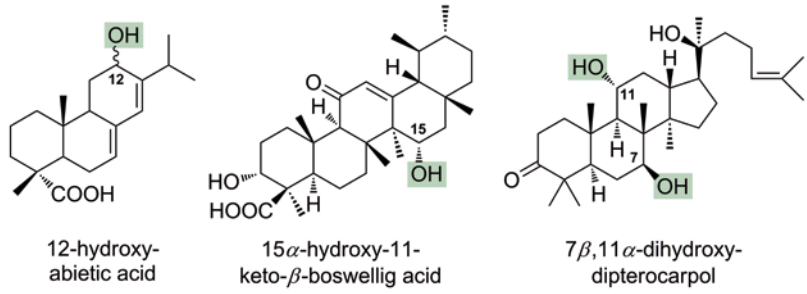

Fig. 8.2 Regioselective hydroxylations of di- and triterpenes catalyzed by CYP106A2

Another bacterial enzyme is CYP106A2 from *Bacillus megaterium:* Although the physiological role of this bacterial P450 is not known, CYP106A2 has been reported to convert a variety of "unnatural" substrates, especially di- and triterpenes, usually with high regio- and stereoselectivities. By screening of a library containing 16,671 synthetic organic compounds, Rita Bernhardt and coworkers identified several compounds of high commercial interest that are oxidized by CYP106A2 (Fig. 8.2).

Reactions include the regioselective allylic C12-hydroxylation of the plant diterpene abietic acid leading to 12-α- and 12-β-hydroxy-abietic acid [88], C15-hydroxylation of the pentacyclic triterpene 11-keto-β-boswellic acid [89], as well as C7- and C11-hydroxylation of the triterpenoid dipterocarpol leading to 7-β,11-α-dihydroxy-dipterocarpol [90]. All hydroxylated products could be produced by recombinant expression of CYP106A2 in *Bacillus megaterium* strains. Utilizing the recombinant whole-cell biocatalysts, final yields of 64 mg 12-hydroxy-abietic acid and 33 mg of 7-β,11-α-dihydroxy-dipterocarpol (within 48 h) as well as 561 mg L^{-1} day^{-1} 15-α-hydroxy-11-keto-β-boswellic acid were achieved.

Another enzyme that has recently been described to catalyze highly selective oxidations of diterpenoids is the well-characterized CYP105A1 from *Streptomyces griseolus* [91]. By screening of a small compound library consisting of the eight most abundant diterpene resin acids of the abietane and pimarane type, all compounds were found to be oxidized by CYP105A1. Oxidations of three substrates, namely abietic acid, dehydroabietic acid, and isopimaric acid, were highly specific, yielding exclusively one product. In the case of abietic and dehydroabietic acid, they were identified as 15-hydroxy-abietic acid and 15-hydroxy-dehydroabietic acid, respectively. The pimarane-type isopimaric acid, which lacks the isopropyl function in favor of a methyl and vinyl group at C13, was converted to 15,16-epoxyisopimaric acid (Fig. 8.3). The hydroxylation of abietic acid at C15 is extremely interesting, because the easy aromatization of ring carbon atoms is a major hurdle in

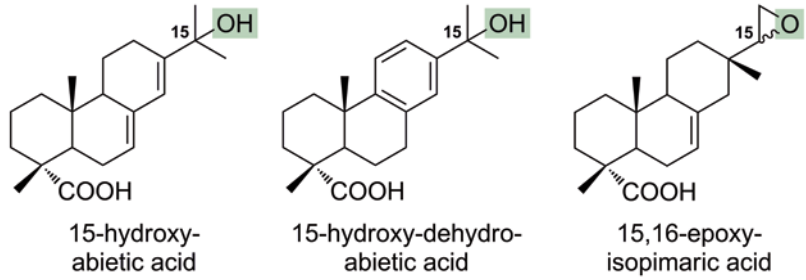

Fig. 8.3 Regioselective hydroxylations of resin acids catalyzed by CYP105A1

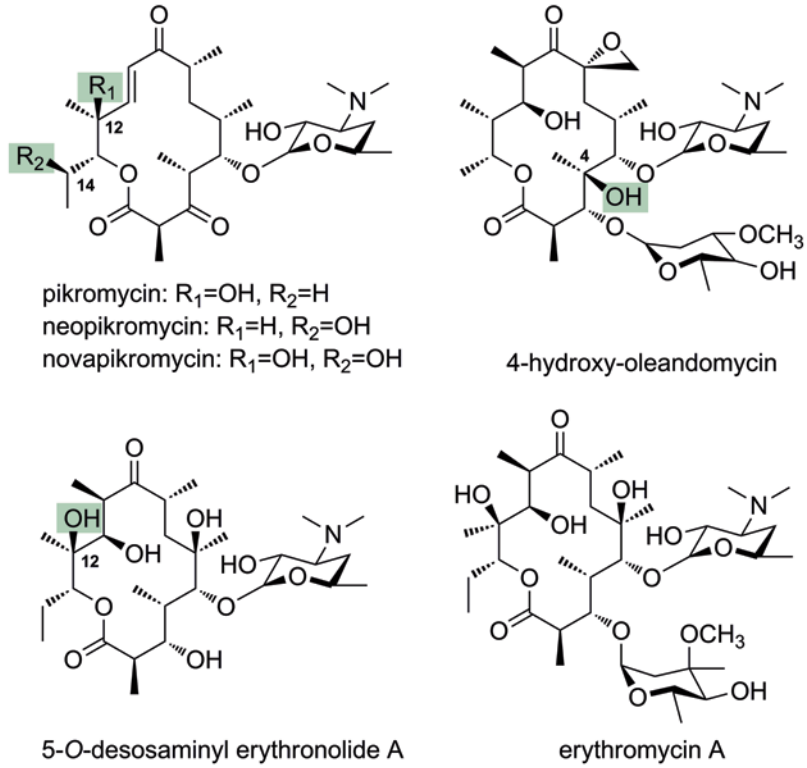

Fig. 8.4 Macrolide antibiotics originating from erythromycin A and their hydroxylated derivatives produced by P450 PikC

chemical synthesis of hydroxylated abietic acid derivatives [91].

The same research group designed an *E. coli* whole-cell biocatalyst expressing CYP105A1. Additionally, an enzyme-coupled cofactor regeneration system was integrated by coexpression of an alcohol dehydrogenase from *Lactobacillus brevis*. After optimizing the expression and conversion conditions, the cells were able to completely convert 200 μM of abietic acid into 15-hydroxy-abietic acid within 2 h [92].

8.2.4 Selective Oxidations of Macrolide Antibiotics

Macrolides belong to the class of polyketides. Their core structure is synthesized by polyketide synthases based on general precursor molecules and then further diversified among other P450-catalyzed hydroxylations and epoxidations [93].

Thirty five percent of all marketed antibiotic formulations contain an active ingredient derived from an actinomycete; since most antibiotics are semisynthetic derivatives of a few natural products, actinomycetes produce an impressive 76 % of all original natural product scaffolds used as anti-infective agents [94]. Therefore, the "deorphanization" of actinomycetes P450s is considered quite important for pharmacology, with ramifications for the use of clinical therapeutics [95, 96]. Heterologous gene expression is one of the main strategies used to access the full biosynthetic potential of *Streptomyces*, as well as to study the metabolic pathways of natural product biosynthesis and to create unnatural pathways [94].

A well-characterized P450 involved in ring decoration of macrolide antibiotics is PikC from *Streptomyces venezuelae* catalyzing regioselective C12-hydroxylation of narbomycin—the final step in pikromycin biosynthesis (Fig. 8.4) [97]. PikC is also involved in the production of

neopikromycin (arising from C14-hydroxylation of narbomycin) and novapikromycin (arising from C14-hydroxylation of pikromycin) [98]. Furthermore, PikC was demonstrated to perform regio- and stereoselective C4-hydroxylation of oleandomycin [99], as well as regioselective C12-hydroxylation of 5-*O*-desosaminyl erythronolide B yielding 5-*O*-desosaminyl erythronolide A (Fig. 8.4) [100].

MycCI and MycG were found in the mycinamicin biosynthetic gene cluster of *Micromonospora griseorubida*. MycCI catalyzes the C21-hydroxylation of mycinamicin VIII yielding mycinamicin VII. In the biosynthetic pathway, the substrate mycinamicin IV undergoes consecutive hydroxylation and epoxidation by the dual-functional P450 MycG, yielding the final product mycinamicin II [101].

Several reports on the identification and characterization of other macrolide-modifying P450s exist—for example, CYP154C1, 105D6, 105D7, 105F2, 105P1, or 170A1—and have been reviewed [96, 102, 103].

8.3 Protein Engineering of P450s

8.3.1 General Strategies

Protein engineering, the process of developing useful proteins for a certain target, has been widely applied to generate P450s with altered substrate specificities, substantially increased activities, and/or enhanced process stabilities. The large number of studies on P450 engineering not only provides new biotechnologically relevant catalysts but also leads to fundamental insights on how changing certain features of the active site of an enzyme might influence its properties. Several comprehensive reviews on P450 engineering have been published recently [4, 104–108]. Outstanding in this regard is the review by Whitehouse et al. [109] summarizing almost all reports on CYP102A1 (also referred to as P450 BM3) from *Bacillus megaterium* with the aim of creating a resource that can be used as a gateway to the field.

There are two general strategies for protein engineering (Fig. 8.5) [110]:

1. "Rational protein design" based on structural knowledge of the protein of interest and computational modeling; and
2. "Directed evolution", which resembles the process of natural evolution and in principle can be applied without knowledge of the protein structure or even the DNA sequence.

Directed evolution experiments use random (point) mutagenesis of a whole gene or domain, insertions and deletions, as well as other, more hypothesis-driven mutagenesis schemes [111]. Another important natural mutation mechanism is recombination of homologous genes, which is highly conservative as compared to random mutation (Fig. 8.5). Thus, a protein can acquire numerous mutations by recombination and still retain its function, whereas similar levels of random mutation may lead to loss of function [111]. The major limitation of directed evolution is, however, that it requires the screening of large variant libraries with thousands of clones. In most cases, the hit rates for new activities are rather low.

In recent years, many P450 proteins (either with or without substrates) were crystallized and their structures were solved, which greatly aided the rational design of these enzymes. For example, a search for the term "P450" in the *P*rotein *D*ata *B*ank database (PDB; http://www.rcsb.org/pdb/home/home.do; 2014/03/27) resulted in 712 structure hits of 47 individual P450 enzymes. Particularly interesting are protein structures of P450 enzymes in their "productive" conformation, e.g., with a C–H bond of the substrate close to the heme iron, as they help to explain (at least to some extent) P450 catalysis and their ability to accept a large variety of substrates. There are, however, still only few such structures available.

A disadvantage of rational design is that even if a crystal structure of a P450 is available, the number of potential substrate-interacting residues is often quite high and therefore an exhaustive analysis of possible cooperative effects is required.

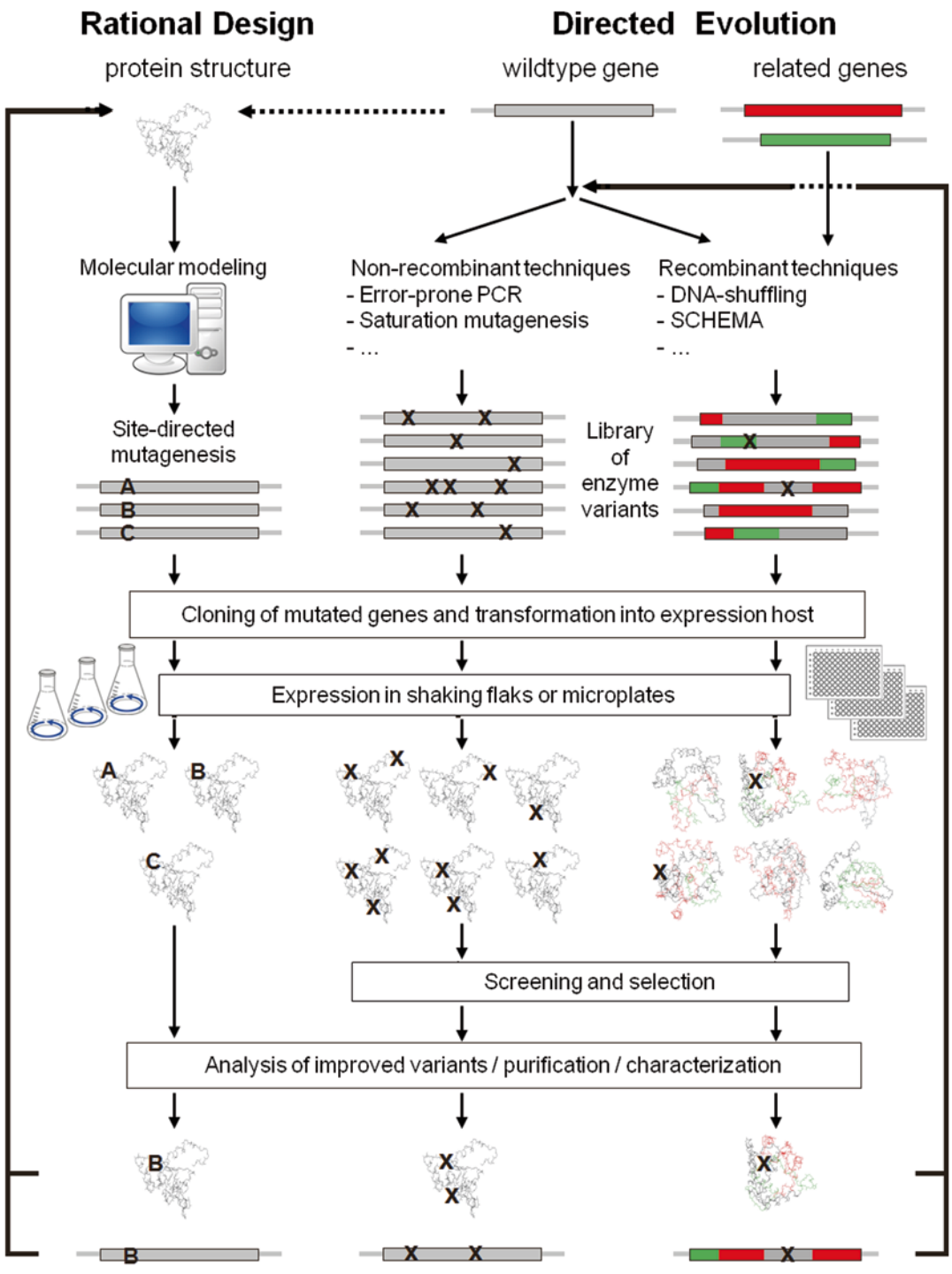

Fig. 8.5 General strategies applied for protein engineering. While "rational design" is based on structural knowledge of the protein and computational modeling, "directed evolution" can be applied without such knowledge

Taken together, directed evolution and rational design are not mutually exclusive, and novel techniques for P450 engineering often combine both strategies. Facilitated by accumulating knowledge of P450 structures and function, as well as advances in (automated) high-throughput technology, the capabilities of P450 engineering are greatly expanding.

8.3.2 Selected Examples of P450 Engineering

A vast number of reports on P450 engineering is currently available. In this section, several selected examples of P450 engineering are discussed in detail, with special attention to altering the P450 substrate spectrum and selectivity.

8.3.2.1 Altering the Substrate Spectrum of P450s

Xenobiotic-metabolizing hepatic P450s accept a broad range of substrates but display low activities and are difficult to express in recombinant hosts. In contrast, many bacterial P450s usually demonstrate narrower physiological substrate profiles but they are easier to handle. Therefore, altering the substrate scope of bacterial P450s to accept nonphysiological substrates is an obvious target of protein engineering. P450 engineering via evolutionary approaches has provided a major route towards this goal [4].

P450 BM3 represents an obvious target for engineering, largely because of its high catalytic activity, solubility, and high expression level in *E. coli,* as well as the early availability of structural information of the monooxygenase domain. The wildtype enzyme catalyzes the hydroxylation of linear and branched fatty acids with a chain length of C12–C20 at subterminal (ω-1, ω-2, ω-3) positions with high turnover rates (1000–3500 min^{-1}, or even 17,100 min^{-1}, for arachidonic acid [112]) and high coupling efficiencies of 88–98% [113, 114].

P450 BM3 has been engineered for oxidation of alkanes, terpenes, heteroaromatics, alkaloids, steroids, and other classes of chemical substances, catalyzing hydroxylations, epoxidations, and demethylations [109]. In some cases, turnover rates and coupling efficiencies of the P450 BM3 variants were comparable to those measured for the wildtype enzyme with fatty acids. For example, a laboratory-evolved P450 propane monooxygenase ($P450_{PMO}R2$) with 20 heme domain substitutions oxidized propane with turnover rates of 370 min^{-1} and a coupling efficiency between NADPH and substrate oxidation of more than 98%. More importantly, a total turnover number of 45,800 could be achieved with this variant [115].

The P450 BM3 variant A74G/F87V/L188Q designed by saturation mutagenesis was shown to oxidize indole, *n*-octane, highly branched fatty acids and fatty alcohols, polychlorinated dibenzo-*p*-dioxins, polyaromatic hydrocarbons, styrene, and many other chemical compounds [116–120]. The monoterpene geranylacetone was converted by P450 BM3 R47L/Y51F/F87V with high activity (>2000 min^{-1}) and stereoselectivity (97% *ee*) to the single product 9,10-epoxygeranylacetone [121].

Although wildtype P450 BM3 has not been able to metabolize any drug-like compound tested so far, it has been turned by rational protein design and directed evolution into an enzyme that oxidizes human drugs, such as testosterone, amodiaquine, dextromethorphan, and 3,4-methylenedioxymethyl-amphetamine [122], as well as propranolol [123] and buspirone [124]. Other P450 BM3 variants with applicability as biocatalysts in the production of reactive metabolites from the drugs clozapine, diclofenac, and acetaminophen [125], as well as variants metabolizing trimethoprim—an antibacterial agent [126]—have also been reported. Several examples hereof are discussed in Sect. 8.6.

Another major target for protein engineering is CYP101A1 (also referred to as P450cam) from *Pseudomonas putida*. The wildtype enzyme catalyzes the regioselective and stereoselective oxidation of (+)-camphor to 5-*exo*-hydroxycamphor with a turnover rate of >2000 min^{-1} and a coupling efficiency of >95% under optimal conditions, i.e., in the presence of saturating concentrations of its physiological redox partners putidaredoxin reductase (PdR) and putidaredoxin (Pdx) [127].

P450cam has been engineered, primarily via structure-based rational design, to accept a variety of nonnative substrates such as other terpenes (e.g., (+)-α-pinene), alkanes, diphenylmethane, styrene, polychlorinated benzenes, and other aromatic compounds (reviewed in [4]). These studies revealed four "hot spots" that determine the substrate specificity of P450cam, namely the residues Y96, F87, L244, and V247.

How far protein engineering can be driven is demonstrated by the structure-based engineering of P450cam variants that are able to convert propane to propanol with a turnover rate of 176 min^{-1} and a coupling efficiency of 66% (F87W/Y96F/T101L/L1244M/V247L; named EB-variant) [128], or ethane to ethanol with a turnover rate of 78 min^{-1}, albeit with only 10% coupling efficiency (EB-variant + L294M/T185M/L1358P/G248A) [129].

Another strategy to alter the substrate spectra of P450s is the creation of chimeras, which has been applied to bacterial and mammalian P450s. Common approaches for the generation of chimeras are "DNA-shuffling" (e.g., as applied for the CYP2C subfamily [130, 131] or CYP11A1 [132]), computationally guided recombination (e.g., SCHEMA, as described below), or the exchange of substrate recognition sites (SRS) across unrelated P450s (e.g., chimeras of P450 BM3 and CYP4C7 [133, 134]).

An approach called SCHEMA, which is based on structure-guided DNA recombination, was developed and applied by Frances Arnold and colleagues to obtain chimeras containing the heme-binding domains of P450 BM3 and its homologues CYP102A2 and CYP102A3, sharing only ~60% amino acid identity [135]. A survey of the activities of the new P450 chimeras revealed completely novel functions that were absent in the wildtype enzymes. These functions included the ability to accept and oxidize drugs like verapamil and astemizole [136].

Highly active chimeric fusion proteins were constructed by swapping reductase domains of several P450 BM3 mutants. Subsequently, random mutagenesis at the heme domain of the chimeras was applied to generate chimeric variants that were more active than the parental chimeras. Some of the chimeric variants showed high activity towards typical human P450 substrates including drugs [137].

8.3.2.2 Altering the Selectivity of P450s

A challenging problem for P450 engineering is the fine-tuning of regio- and/or stereoselectivities. Generally, mutations that are intended to expand the substrate spectrum of a P450 towards nonphysiological compounds will typically enlarge the active site where substrate docking occurs. This often allows the substrate of interest to bind in multiple orientations resulting in poor regio- and/or stereoselectivities. In addition, high-throughput screenings are usually not feasible for determination of regio- and stereoselectivity, but instead must be established on a case-by-case basis, e.g., through (chiral) gas chromatography (GC)- or liquid chromatography (LC)-analysis [4]. Nevertheless, successful alterations of the regio- and/or stereoselectivity of P450s have been reported.

For example, the regioselectivity of CYP106A2 could be altered considerably by site-saturation mutagenesis. CYP106A2 is a bacterial steroid hydroxylase that hydroxylates inter alia progesterone to 15-β-hydroxy-progesterone, as well as 11-α-hydroxy-progesterone, 9-α-hydroxy-progesterone, and 6-β-hydroxy-progesterone as minor products. Based on homology modeling and substrate docking experiments, the residues A395 and G397 in the active site were identified as possible candidates contributing to enzyme regioselectivity [138]. Saturation mutagenesis combined with subsequent library screening has revealed the variants A395I and A395W/G397K with high 11-α-hydroxylase activity [139].

The systematic comparison of 29 P450 crystal structures and more than 6300 P450 sequences has revealed conserved structural elements in close proximity to the active heme oxygen that are important for the interaction with any given substrate [140]. Based on this study, a minimal P450 BM3 variant library of only 24 variants was constructed by exchanging the amino acids in positions 87 and 328, located in the identified region, for one of the five hydrophobic amino acids (A, V, F, L, or I). The library was screened

with four terpene substrates: geranylacetone, nerylacetone, (4*R*)-limonene, and (+)-valencene. As compared to the wildtype enzyme, most variants demonstrated either a strongly shifted or improved regio- or chemoselectivity for the oxidation of at least one substrate [141]. Members of this library also exhibited an up to 100-fold higher hydroxylation activity towards cyclooctane and cyclodecane. Furthermore, several variants were identified that hydroxylated cyclododecane, a reaction that cannot be catalyzed by the wildtype enzyme [142]. The main advantage of this iterative approach compared to site-directed mutagenesis is that, through the specific choice of two amino acids located close to each other, unpredictable synergistic effects can be achieved.

Another iterative approach called "combinatorial active-site saturation test (CAST)" was successfully applied to engineer P450 BM3 variants with high regio- and stereoselectivity for testosterone and progesterone oxidation [143]. Twenty active-site positions, identified using the three-dimensional structure of P450 BM3 and based on earlier studies, were divided into nine groups in order to maximize the probability of cooperative effects within a single site and between different sites. While the parent P450 BM3 F87A variant produces a mixture of 2-β- and 15-β-hydroxytestosterone, simultaneous substitutions at the positions R47/T49/Y51 provided a variant, yielding up to 94% of 2-β-hydroxytestosterone. In contrast, combined mutations in the sites V78 and A82 favored the 15-β position for hydroxylation. Further mutagenesis including these two positions increased the regio- and stereoselectivity of the final variant R47Y/T49F/V78L/A82M/F87A up to 96% towards 15-β-hydroxylation [143].

8.3.2.3 Miscellaneous

A unique approach to expand substrate spectra of P450s to nonnatural compounds without mutagenesis or substrate engineering has been described [144]. Substrate-like "decoy molecules" were employed to extend the substrate spectrum of the natural fatty acid peroxygenase CYP152A1 ($P450_{Bs\beta}$) from *Bacillus subtilis*. The decoy molecule (a short chain fatty acid) is able to bind in the binding pocket but is not converted by the enzyme [144]. It was suggested that the carboxylate group of the decoy molecule serves as the general acid–base catalyst, which is indispensable for the efficient generation of the active P450-species using H_2O_2 [145]. By using this approach, guaiacol, styrene, and ethylbenzene were successfully oxidized by CYP152A1 [144].

The same strategy was later applied to P450 BM3 for the hydroxylation of gaseous alkanes [146]. Herein, perfluorocarboxylic acids with chain length between C8 and C14 that bind in the binding pocket with increasing affinity, served as the decoy molecules. Propane, butane, and cyclohexane were subsequently used as substrates. As products 2-propanol, 2-butanol, and cyclohexanol, respectively, were formed. Interestingly, only the enzyme activity but not its regioselectivity upon octane oxidation changed in the presence of perfluorononanoic acid [147].

8.3.3 Molecular Tools for the Construction of P450 Fusionproteins

From a biotechnological point of view, the main focus of P450 engineering was initially on the heme-containing P450 domain to enhance activity or selectivity as described above. It was quickly noted, however, that the dependence of P450s on accessory redox partners and the requirement for NAD(P)H limits their biotechnological exploitation (discussed in detail in [148]). To circumvent these limitations, different approaches for efficient delivery of electrons to the heme of P450s have been developed. These include among others, enzymatic cofactor regeneration, chemical or electrochemical reduction of P450s, and photochemical (light-driven) regeneration of P450s. These approaches will be described in Sect. 8.5.

An alternative engineering strategy is represented by the generation of man-made fusions of redox partners with P450 enzymes (described in detail in Sect. 8.4). To date, a variety of molecular tools have been developed to optimize redox chains and to facilitate the construction of artificial P450–redox partner fusion enzymes:

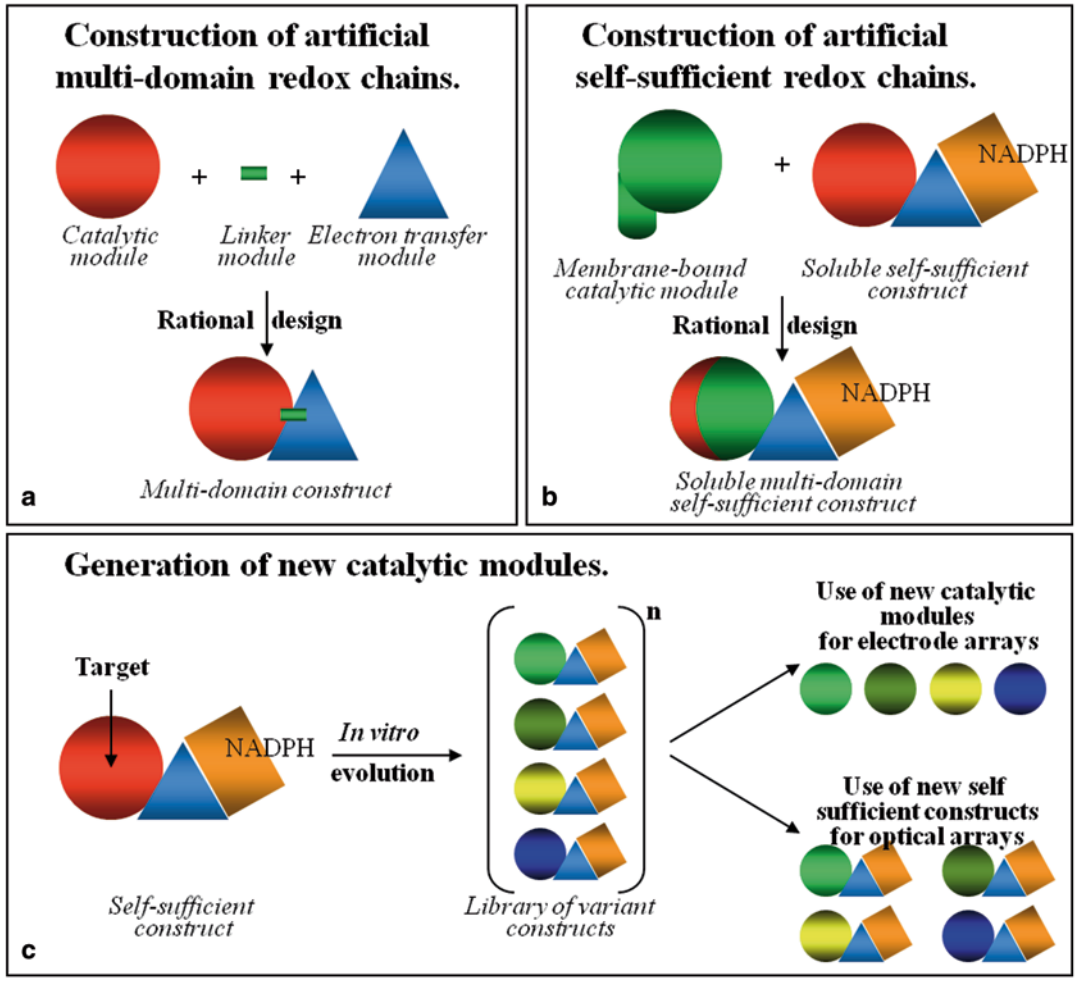

Fig. 8.6 The molecular Lego approach applied to the scaffold of P450 BM3; **a** to generate a P450 catalytic domain electrochemically accessible through the fusion with the electron transfer protein flavodoxin; **b** to solubilize the human membrane-bound P450 2E1 by fusion with selected parts of the scaffold of the catalytically self-sufficient P450 BM3; **c** to generate libraries of P450 BM3 enzymes with different catalytic domains to be used for pharmacological and biosensing applications. (Reproduced from [149] with permission of Elsevier Limited, Oxford, UK)

1. "Molecular Lego": An approach for the design of molecular assemblies of P450 enzymes and redox partners for nanobiotechnology [149, 150].
2. "LICRED": A versatile drop-in vector for rapid generation of redox-self-sufficient cytochrome P450s [151, 152].
3. "PUPPET": A protein scaffold-based approach (*P*CNA-*u*tilized *p*rotein complex of *P450* and its two *e*lectron *t*ransfer-related proteins) [153, 154].

8.3.3.1 Molecular Lego

The "molecular Lego" approach for the construction of artificial P450 fusion enzymes was developed with the aim to generate P450 enzymes with novel catalytic functions (Fig. 8.6) [149]. In analogy to the children's toy "Lego", monooxygenase domains and reductase domains of different P450 systems were used as building blocks for the construction of catalytically self-sufficient enzymes.

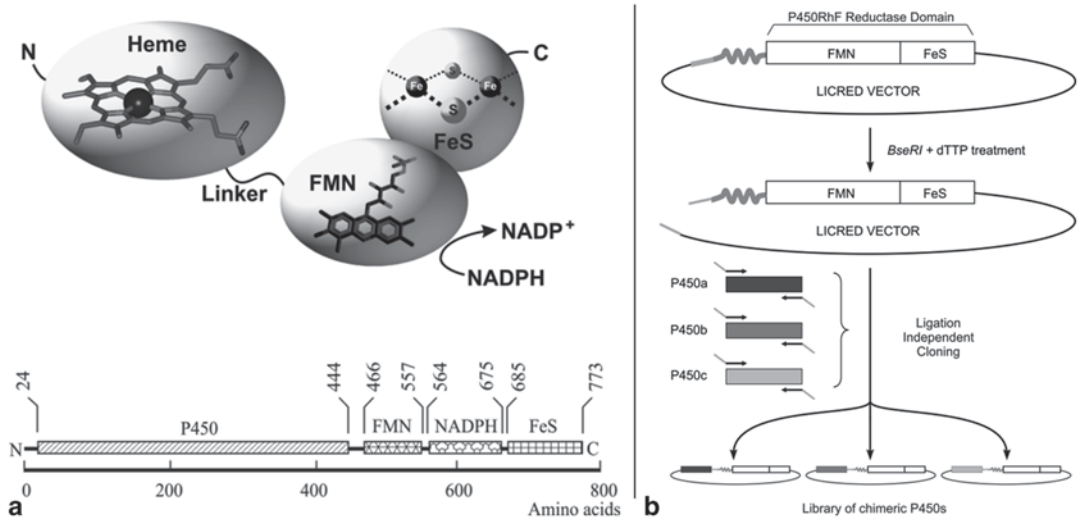

Fig. 8.7 LICRED. **a** Schematic representation of the architecture of P450RhF (CYP116B2) spanning from the N-terminal heme domain to the C-terminal reductase domain. **b** Schematic representation of the general strategy used to clone P450 heme domains in the LICRED platform to generate libraries of self-sufficient P450s. (Reproduced from [152] with permission of WILEY-VCH, Weinheim, Germany)

This approach proved to be a powerful molecular tool, especially in combination with error-prone polymerase chain reaction (PCR) to generate a library of random variants of P450s and subsequent screening for P450 activity by an in-house developed alkali-based method [149]. Gianfranco Gilardi and coworkers constructed a fusion protein comprised of an N-terminal human CYP2E1 module (residues 22–439) and a C-terminal reductase module containing the reductase domain of P450 BM3 (residues 473–1049) [155]. The CYP2E1 module lacked the hydrophobic N-terminus, which permitted expression of the CYP2E1–BMR fusion enzymes in soluble form [155]. CYP2E1–BMR was shown to be catalytically self-sufficient and to exhibit many of the hallmarks of CYP2E1, including catalytic activity towards the typical substrates *p*-nitrophenol and chlorzoxazone. CYP2E1–BMR catalyzed the hydroxylation of *p*-nitrophenol with a k_{cat} of ~3 $nmol_{product}$ min^{-1} $nmol_{P450}{}^{-1}$, whereas with chlorzoxazone, a k_{cat} of ~1 $nmol_{product}$ min^{-1} $nmol_{P450}{}^{-1}$ was measured. Importantly, CYP2E1–BMR achieved wildtype-like activities without the addition of detergents and lipids [155].

8.3.3.2 LICRED

The ever-growing number of discovered P450s calls for high-throughput tools to facilitate their isolation and characterization. For this purpose, the ligation-independent cloning (LIC) vector termed "LICRED" (Fig. 8.7) was designed to facilitate the high-throughput construction of libraries of catalytically self-sufficient P450 fusion enzymes by connecting a variety of monooxygenase domains to the reductase domain of P450RhF (RhFRed) of the self-sufficient P450RhF (CYP116B2) from *Rhodococcus* sp. Target P450s are amplified with specifically designed PCR primers containing LIC-compatible overhangs that allow for cloning into the LICRED vector [151, 152].

In such manner, fusion enzymes comprised of RhFRed and the monooxygenase domains of either P450cam or CYP177A1 were successfully produced [152]. These artificial P450 systems were shown to be expressed in a soluble form and to be catalytically active. Importantly, electrons from NADPH were shown to be transferred primarily intramolecularly to the P450 heme domain. The robustness and universal applicability of LICRED was demonstrated by generating a

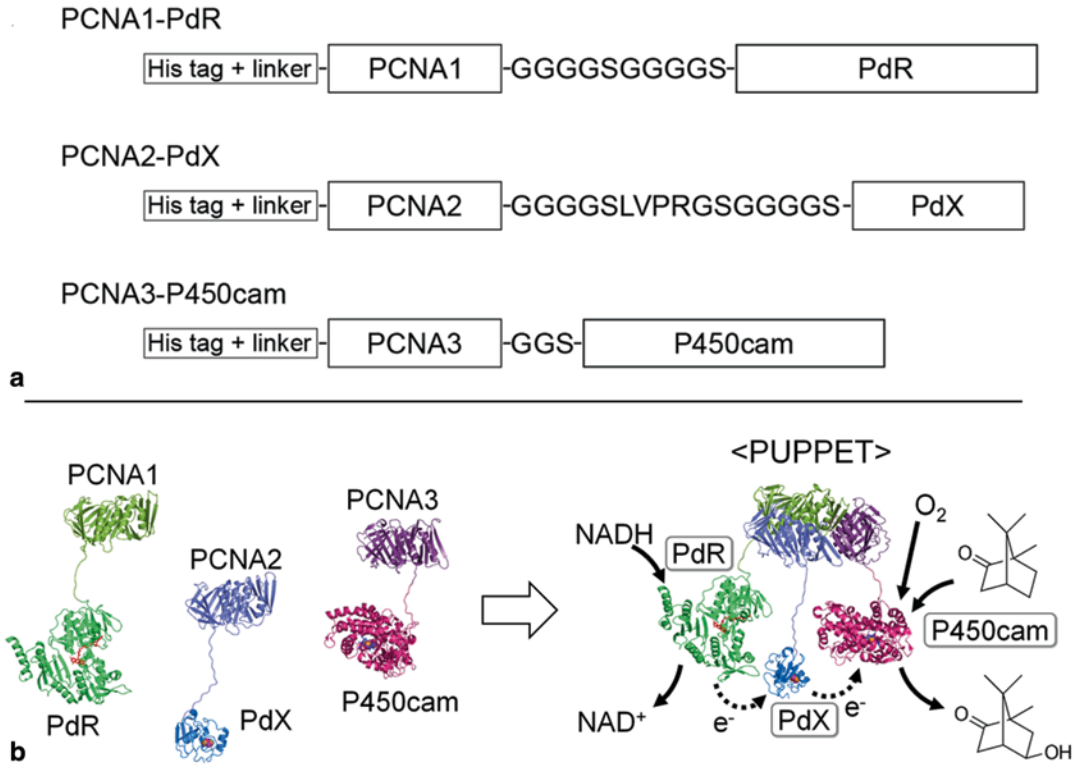

Fig. 8.8 *P*CNA-*u*tilized *p*rotein complex of *P450* and its two-electron-*t*ransfer-related proteins (PUPPET). **a** Schematic representation of the link design of individual PCNA fusion proteins. **b** Model depicting the self-assembly of PCNA1-PdR, PCNA2-PdX, and PCNA3-P450cam. (Reproduced from [153] with permission of WILEY-VCH, Weinheim, Germany)

library of RhFRed fusion constructs with 22 different P450s taken from the genome of *Nocardia farcinica*. Subsequent screening of this library against a variety of substrates identified fusion enzymes that were capable of the hydroxylation of testosterone and methyltestosterone, as well as dealkylation of 7-ethoxycoumarin [152].

8.3.3.3 PUPPET

Besides constructing "linear" P450–redox partner fusion enzymes, alternative approaches have been followed to bring the redox partners and P450 monooxygenase in close proximity to each other for efficient electron transfer. Inspired by the high stability and high catalytic activity of multienzyme complexes occurring in nature, Teruyuki Nagamune and coworkers created a platform that employs three distinct proliferating cell nuclear antigens (PCNAs) from *Sulfolobus solfataricus* that assemble into a heterotrimer [153, 154]. A heterotrimeric complex called "PUPPET" was created that consisted of PCNA1-PdR, PCNA2-Pdx, and PCNA3-P450cam (Fig. 8.8) [153].

PUPPET exhibited a much higher monooxygenase activity than control reaction mixtures containing equimolar amounts of PdR, Pdx, and P450cam [153]. The authors suggested that the close proximity of P450cam to its dedicated redox partners fused to the PCNA scaffold allowed more efficient electron transfer from PdR to P450cam via Pdx [153]. Moreover, this system was recently further optimized by replacing the GGGGSLVPRGSGGGS linker connecting PCNA2 and Pdx, by the more rigid, proline-rich linker GGGGS$(PPPPP)_4$GGGGS, which improved the monooxygenase activity of the system by almost twofold [154]. Likely, the rigid stretch

Table 8.2 Classification of P450 systems based on their dedicated redox chains according to Hannemann et al. [35]. (Reproduced with permission of Elsevier limited, Oxford, UK)

P450 class and origin	Electron transfer chain	Typical P450 representatives
Class I *Bacteria, mitochondria*	NAD(P)H → FdR → Fdx → P450	*Pseudomonas putida* CYP101 (P450cam) [162, 163] Mammalian CYP11A1 (P450ssc) [164]
Class II *Bacteria, microsome, plants, fungi*	NAD(P)H → CPR→ P450	*Streptomyces carbophilus* CYP105A3 (P450sca) [165] Microsomal P450s [166]
Class III *Bacteria*	NAD(P)H → FdR → Fld → P450	*Cytrobacter braakii* CYP176A1 (P450cin) [167, 168]
Class IV *Bacteria*	Pyruvate, CoA → OFOR → Fdx → P450	*Sulfolobus solfataricus* CYP119 [169]
Class V *Bacteria*	NADH → FdR → [Fdx-P450]	*Methylococcus capsulatus* CYP51 [170]
Class VI *Bacteria*	NAD(P)H → FdR → [Fld-P450]	*Rhodococcus rhodochrous* CYP177A1 (P450 XplA)[171]
Class VII *bacteria*	NADH → [PFOR-P450]	*Rhodococcus* sp. CYP116B2 (P450RhF) [172, 173]
Class VIII *Bacteria, fungi*	NAD(P)H → [CPR-P450]	*Bacillus subtilis* CYP102A1(P450 BM3) [174–177]*Fusarium oxysporum* CYP505A1 (P450foxy) [178, 179]
Class IX *Fungi*	NADH → [P450]	*Fusarium oxysporum* CYP55 (P450nor) [180]
Class X *Plants, mammals*	[P450]	Plant divinyl ether synthase (DES) (CYP74D) [160] Mammalian thromboxane synthase (TXAS) [159]

FdR ferredoxin/flavodoxin reductase, *Fdx* Ferredoxin, *CPR* cytochrome P450 diflavin reductase, *Fld* flavodoxin, *OFOR* 2-oxoacid-ferredoxin oxidoreductase, *PFOR* phthalate-family dioxygenase reductase

of 20 consecutive proline residues contributes to positioning Pdx close to the Pdx-binding site of P450cam, thereby facilitating electron transfer [154].

8.4 Optimization of Redox Chains

8.4.1 Redox Partners of Cytochrome P450 Monooxygenases

As mentioned above, cytochrome P450 monooxygenase activity relies on the consecutive delivery of two electrons to enable the reduction of the P450 heme iron and the final formation of the highly reactive ferryl-oxo species [156]. These electrons are usually donated by the cellular cofactors NADH or NADPH and are delivered to the P450 monooxygenase by dedicated redox proteins or redox domains. Whereas it is generally believed that the various P450s have evolved from a common ancestor, differences exist in the nature of the electron carriers that deliver the electrons to the P450s [157]. Initially, two main systems for delivery of electrons to P450s were described, namely electron transfer by the cooperative action of ferredoxin reductase (FdR) and ferredoxin (Fdx) for class I P450s, or electron delivery by the flavin adenine dinucleotide (FAD)- and flavin mononucleotide (FMN)-binding cytochrome P450 diflavin reductase (CPR), as is the case for class II P450s [158].

To date, various other routes of electron delivery to P450s have been recognized, leading to diverse classifications, e.g., those suggested by Hannemann et al. (Table 8.2) [35]. The diversity of the P450 systems is striking, ranging from complex systems composed of three individual proteins (classes I, III, and IV) to the more simple catalytic self-sufficient systems that harbor the

necessary redox modules and the P450 monooxygenase enzyme in a single polypeptide chain (classes VII and VIII). P450 fusion enzymes which combine a P450 moiety with either Fdx or a flavodoxin (Fld) moiety were assigned to classes V and VI, respectively. Moreover, P450s have been identified that can function independent of redox proteins and NAD(P)H (class X) [159–161].

To drive monooxygenase activity, human P450s receive the necessary electrons from individual redox partner proteins, which include a CPR or an adrenodoxin reductase–adrenodoxin (AdR–Adx) couple. On the other hand, in bacteria and lower eukaryotes, a number of natural fusions of P450s with their dedicated redox partners have been identified (Fig. 8.9). By comparison, such P450 fusion enzymes usually exhibit much higher turnover rates than the human P450s, which has been attributed, in part, to superior electron transfer within these P450 fusion enzymes [112].

P450 BM3 represents the first natural fusion enzyme that was discovered to harbor both a P450 moiety and a redox partner module within a single polypeptide chain [176, 177]. P450 BM3 is a soluble enzyme that consists of an N-terminal P450 domain exhibiting fatty acid hydroxylase activity, which is fused via a short peptide linker to a C-terminal diflavin-containing CPR-like domain (Fig. 8.9). Thus, P450 BM3 is a self-sufficient P450 system that requires only its substrates, NADPH, fatty acids, and dioxygen for catalytic activity [176, 177].

P450 BM3 has attracted great attention as a model system for biotechnological exploitation due to its unprecedented high enzymatic activity in conjunction with highly efficient electron transfer [109, 181, 182]. In the last decade, an increasing number of P450–redox partner fusion proteins has been identified [170, 172, 183]. Even though a wealth of novel P450 systems have been discovered and constructed since, the highest reported oxygenase activity of a P450 to this day remains that of P450 BM3, with a k_{cat} of 17,100 min^{-1} in the presence of arachidonic acid as a substrate [112].

8.4.2 Linker Design in Protein Engineering

Recent advances in the field of protein engineering have come from constructing multifunctional recombinant fusion proteins [184, 185]. It has been recognized that the proper design of linker peptides is vital to the desired function of the fusion protein.

A systematic study of inter-domain linkers occurring in natural fusion proteins revealed the existence of two main types of linkers: helical and nonhelical [184]. Helical linkers are thought to act as rigid spacers separating two domains. Nonhelical linkers were found to contain a high number of proline residues, which also provides structural rigidity and contributes to isolation of the linker from the connected domains [184]. Based on structural data of more than 600 multidomain proteins, approximately 1300 linker peptides were identified, which were demonstrated to have an average length of 10.0±5.8 amino acids and to exhibit a general preference for the amino acids Pro, Arg, Phe, Leu, Glu, and Gln (in respective order of decreasing preference) [184]. Whereas Pro, Thr, and Phe were dominant in nonhelical linkers, helical linkers were enriched in Leu, Arg, Glu, Met, and Gln [184]. The high preference for Pro in nonhelical linkers was explained by the fact that this residue has no amide hydrogen to donate in hydrogen bonding and therefore reduces the interaction between the linker and the adjacent protein domains [184]. Moreover, it was noted that long linkers (21.0±7.6 residues) exhibited a decreased preference for the hydrophobic amino acid Met, whereas an increased propensity for Cys, Asn, and Gln was observed [184]. In contrast, short linkers (4.5±0.7 residues) showed an increased preference for hydrophobic amino acids and a concomitant decrease in the content of polar and acidic amino acids [184]. It is reasonable to assume that with increasing linker length also the degree of exposure of the linker to solvent is increased, and therefore, longer linkers are more likely to contain hydrophilic amino acids.

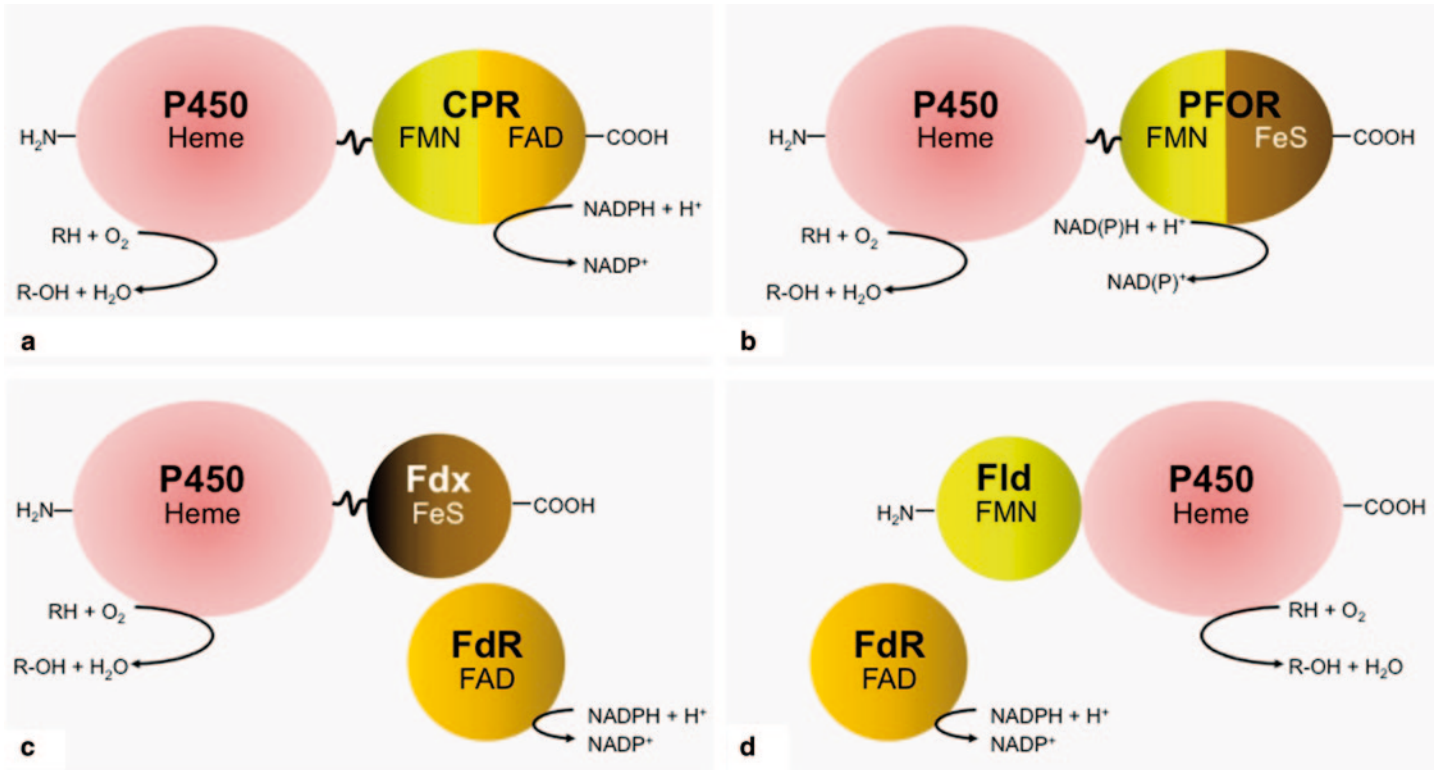

Fig. 8.9 Schematic representation of the four major types of natural P450–redox partner fusions and their domain arrangements. **a** P450 connected via a short flexible linker to a diflavin CPR, exemplified by P450 BM3. **b** P450 fused to PFOR with a 16-amino acid linker, as found in CYP116B2 (P450 RhF). **c** P450 fused via an alanine-rich linker to Fdx and separate FdR, exemplified by the CYP51 system. **d** P450 fused to Fld and separate FdR with CYP177A1 (P450 XplA) as prototype

In many cases, the aforementioned inherent properties of natural linkers of multidomain proteins have served as a basic guide to linker design for the construction of man-made fusion enzymes. For the engineering of recombinant fusion proteins, three main types of linkers are often employed; these include flexible linkers, rigid linkers, and linkers that can be cleaved, either proteolytically or chemically [185].

8.4.2.1 Flexible Linkers

Flexible linkers are often employed in cases where the joined domains or proteins require a certain degree of movement or interaction. Such linkers frequently contain small, nonpolar amino acids such as Gly and/or polar residues such as Ser or Thr [185, 186]. The small size of these amino acids confers flexibility to the linker, which in turn facilitates the movement of the attached domains. In addition, the Ser and/or Thr residues contribute to the stability of the linker in aqueous milieus by forming H-bonds with the water molecules [185]. In such manner also unfavorable interactions between the linker and the attached protein domains are prevented [185]. A typical example of a flexible linker that has been widely used for engineering recombinant fusion proteins is the oligopeptide $(Gly–Gly–Gly–Gly–Ser)_n$ [187–190]. Herein, the linker length can be tailored by inserting several copies of this oligopeptide in tandem to achieve the optimal distance between the attached domains [185].

8.4.3 Rigid Linkers

Rigid linkers have also been successfully employed in generating fusion proteins, often to maintain a fixed distance between the connected protein domains [185]. Arai et al. designed a rigid linker consisting of $A(EAAAK)_nA$ ($n = 2–5$) with the aim of separating the domains of a bifunctional fusion protein [191]. This linker was based on an earlier study by Marqusee and Baldwin, who demonstrated that the small peptide $A(EAAAK)_3A$ adopts an α-helical conformation, which was stabilized by the Glu^-Lys^+ salt bridges within each segment [192]. Arai et al. demonstrated that by inserting the linker $A(EAAAK)_nA$ between enhanced blue fluorescent protein (EBFP) and enhanced green fluorescent protein (EGFP), the efficiency of fluorescence resonance energy transfer (FRET) between the two proteins could be regulated [191]. An increase in the number of EAAAK linker segments reduced the FRET efficiency. In contrast, flexible linkers composed of GGGGS segments of similar length were substantially less effective in reducing the FRET efficiency [191]. Thus, the helical segments controlled the distance between EBFP and EGFP more effectively.

A different type of rigid linkers that have been described consists of proline-rich amino acid sequences, such as $(XP)_n$ [184, 185]. Herein, X represents any amino acid, with preference for Ala, Lys, or Glu [184]. For instance, artificial fusion proteins comprised of interferon-γ(IFN-γ) and gp120 of the human immunodeficiency virus were constructed, using $(Ala–Pro)_n$ linkers of different lengths [193]. All fusion proteins actively formed dimers, but full biological activity of IFN-γ was achieved only with the longest linker consisting of 34 amino acids [193].

Taken together, α-helical linkers and proline-rich linkers form rigid structures that are often deployed in cases where the spatial separation of the individual domains is crucial to maintain the stability and/or biological activity of the entire fusion protein.

8.4.3.1 Cleavable Linkers

Cleavable linkers represent a third large category of linkers used to generate recombinant fusion proteins [185]. Such linkers are often designed with the aim to liberate the separate fusion domains for the desired biological activity. Examples of cleavable linkers include linkers that harbor disulfide bridges or recognition sequences for proteases such as Factor Xa, Cathepsin B, or HCV protease [194, 195]. Whereas the covalent linkage of protein domains may have many advantages, including for instance an increased plasma half-life (e.g., albumin- or Fc-fusions), a potential major drawback includes functional interference between the separate domains leading to reduced biological activity of the fusion

protein [185]. Such drawback might be circumvented by chemical or enzymatic cleavage of the linker, thereby separating the functional domains [185].

Whereas cleavable linkers seem less relevant with respect to engineering functional interactions between P450 and redox partner(s), flexible and rigid linkers have been used frequently to construct a variety of artificial fusion proteins with the aim to increase the efficiency of electron transfer and improve the catalytic efficiency of the P450 system.

8.4.4 Construction of Artificial P450 Fusion Proteins

Nearly all of the naturally occurring P450 redox partner fusion systems are soluble enzymes, which notably, can be more easily purified than their membrane-associated multicomponent relatives. In addition, these natural P450 fusion systems appear to have a superior catalytic activity and stability, with P450 BM3 as the best example [181]. Therefore, a much-applied approach to circumvent laborious efforts to reconstitute P450 redox chains from individual proteins involves the creation of artificial P450 fusion enzymes by linking the usually separate redox partners to selected P450 enzymes [148, 162, 196].

The artificial linkage of redox and P450 monooxygenase modules has been frequently accomplished by introducing linker peptides connecting the C-terminus to the N-terminus of the individual components [196]. Linker peptides can be derived from naturally fused P450 systems (e.g., P450 BM3) or are of man-made origin [148]. Alternatively, the linkage of redox modules can be brought about by introducing disulfide-bridges at sites important for redox partner interaction [148, 196].

Taken together, covalent fusions of redox modules and P450s are thought to have several advantages over the parental multicomponent P450 systems; the fused proteins constitute a simplified redox system, both with respect to protein expression and isolation. In addition, it is a widely accepted view that the covalent linkage of redox partners may govern a more efficient electron transport, which, in turn, may improve the catalytic efficiency of the target P450 enzyme. The number of reports concerning the structure and function of man-made P450 fusion enzymes is increasing rapidly and excellent reviews on this topic are available [112, 148, 181, 196]. A variety of fusion proteins containing selected heme domains of mammalian, plant, fungal or bacterial P450s, and redox partner proteins, either from bacterial sources (class II and VIII) or from microsomal origin (class II), were shown to exhibit catalytic activity [148, 196]. Here, for simplicity, only a small selection of developed artificial P450 fusion systems relevant to biotechnological exploitation will be discussed in some detail, with special attention to the different strategies that were employed to generate the various fusion enzymes.

8.4.5 Artificial P450—Redox Partner Fusion Enzymes

8.4.5.1 Eukaryotic Fusions Comprised of P450 and CPR

The first self-sufficient fusion comprising a eukaryotic P450 and a CPR was already reported in 1987 by Murakami et al., who fused rat CYP1A1 to rat CPR. Herein, the P450 moiety was fused with its C-terminus to the CPR lacking its N-terminal membrane anchor [197]. Spectral properties of the fused enzyme confirmed the presence of heme, FAD, and FMN as prosthetic groups. Moreover, the fusion enzyme exhibited monooxygenase activity towards 7-ethoxycoumarin following first-order kinetics [197]. This pioneering study initiated the construction of a large number of microsomal catalytic self-sufficient P450–CPR fusion enzymes that exhibited altered and often improved enzymatic properties.

Following a similar strategy, the same research group constructed a set of seven different fusions between bovine CYP17A1 (P450c17) and yeast CPR, which differed in the length and amino acid composition of the linker region between the P450 and CPR domain, due to differ-

ences in truncation of the N-terminus of CPR [198, 199]. Most of these fusions showed improved 17-α-hydroxylase activity as compared to CYP17A1 control reactions. Moreover, these studies demonstrated that both the length and amino acid composition of the linker region contributed to efficient intramolecular electron transport [198, 199].

Such type of P450 fusion system was further diversified with the biotechnological aim to produce steroidogenic specialty drugs [196]. Heme domains of bovine, guinea pig, or porcine microsomal CYP17A1 have been consequently fused to either rat or yeast CPR [196]. For example, a fusion protein of bovine CYP17A and modified rat CPR, linked via the dipeptide Ser-Thr, yielded a fusion enzyme capable of catalyzing the 17-α-hydroxylation of progesterone and pregnenolone [196, 200]. Similarly, several different fusions of bovine CYP21 and yeast CPR have been produced that were active in the 21-hydroxylation of 17-α-hydroxyprogesterone [199]. The various fusion enzymes differed with respect to the order of the functional domains (i.e., CYP21–CPR vs. CPR–CYP21), as well as the linker region in between [199]. The CYP21–CPR fusion with a Ser–Thr linker showed the highest catalytic activity with a V_{max} of 222 $nmol_{product}$ min^{-1} $nmol_{P450}{}^{-1}$ that was about twofold higher when compared to control reactions where CYP21 and CPR were expressed as separate proteins. It was concluded that the higher catalytic activity was governed by efficient electron transfer via intramolecular interaction of the P450 and CPR domains within the fusion enzyme [199].

8.4.5.2 Plant P450 Fusion Enzymes

The kingdom of plants represents a valuable apothecary, as it is the origin of many important therapeutic agents. Thus, the expression of plant P450 fusion enzymes in bacteria may permit the high-level production of medically relevant compounds that plants produce naturally at low levels. In *Catharanthus roseus,* the synthesis of vinblastine and vincristine, two important alkaloids that find application in the treatment of leukemia, starts with tabersonine hydroxylation [201]. Schröder et al. generated a fusion enzyme of *C. roseus* CYP71D12 linked on the N-terminal to its cognate CPR. The fusion enzyme was successfully expressed in *E. coli* and was demonstrated to catalyze 16-hydroxylase activity of tabersonine [201].

Plant P450 systems may also find biotechnological application in the field of herbicide resistance. For example, CYP71B1 from *Thlaspi arvense* covalently attached to CPR from *C. roseus* was shown to metabolize the polycyclic aromatic hydrocarbon benzo(a)pyrene [202]. For expression purposes, the N-terminus of CYP71B1 was modified to code for the initial eight amino acids of CYP17A [202]. In a subsequent study, it was demonstrated that this fusion protein has potential in bioremediation [203]. The aforementioned fusion protein could be immobilized using an oil-in-water macro-emulsion called polyaphron and was shown to be active in metabolizing the antibiotic erythromycin, as well as the herbicide chlortoluron, with activities superior to those of the free P450 [203]. Similarly, Didierjean et al. generated fusion enzymes of CYP76B1 from *Helianthus tuberosus* with truncated forms of CPR from the same organism, which were able to rapidly catalyze the oxidative dealkylation of various recalcitrant herbicides, including isoproturon and chlortoluron [204].

Further examples of plant P450-CPR fusions are described in Sect. 8.7.

8.4.5.3 Bacterial P450 Fusion Enzymes

Among microbial P450s, P450cam has been a major target for the construction of artificial fusions [148, 196]. As such, P450cam was fused to its natural redox partners (PdR and Pdx) to generate a tripartite catalytic self-sufficient P450 system [205]. In this study, different orders of the individual components, as well as different linker sequences, were tested. The highest camphor turnover (k_{cat} ~30 min^{-1}) was observed with the PdR–Pdx–P450cam fusion enzyme, with peptide sequences TDGASSS and PLEL as linker between the respective components [205]. The authors noted that for their fusion system the order of the components rather than the linker length was critical for catalytic activity. It is of note that reconstitution of the P450cam system from its in-

dividual components at a 1:1:1 ratio still outperformed the aforementioned fusion enzyme [205]. Nevertheless, these results demonstrated the feasibility of constructing P450 fusion enzymes for bacterial bioreactors for metabolizing xenobiotics or synthesis of fine chemicals.

Nodate et al. demonstrated that fusions of P450cam and RhFRed from *Rhodococcus* sp. could be functionally expressed in *E. coli* [206]. To enhance the efficiency of this type of fusion enzyme, Robin et al. generated in a follow-up study a set of seven P450cam–RhFRed fusion constructs using peptide linkers of different lengths [207]. The introduction of a nine-amino-acid linker (HMRLASTHM) between the components accomplished a higher in vivo conversion of (+)-camphor to 5-*exo*-hydroxycamphor, improving the yield 20-fold. By further optimizing the reaction conditions, 80 % conversion was obtained at a substrate concentration of 30 mM, which makes this P450 fusion system amenable to industrial biocatalysis [207].

8.4.5.4 Mixed P450 Fusion Enzymes

Whereas the heme domain of P450 BM3 has been subject to extensive engineering, its reductase domain (BMR), in addition, has been frequently employed as a redox partner in artificial P450 fusion constructs [112]. Fusion constructs between N-terminally modified forms of human CYP2C9, 2C19, and 3A4 and BMR, connected via a Pro–Ser–Arg linker, were all demonstrated to be catalytically self-sufficient and to exhibit turnover rates that were comparable to those obtained for the native human P450s when reconstituted with their natural CPRs [112, 208]. For example, the CYP2C9–BMR fusion enzyme catalyzed the 4-hydroxylation of diclofenac with a k_{cat} of 40 min^{-1} [208].

In a different approach, hybrids of P540 BM3 and neuronal nitric oxide synthase (nNOS) were generated in which the heme and reductase domains of the respective enzymes were swapped, while maintaining the natural domain order [112, 209]. Such hybrids could successfully be expressed in *E. coli* and were shown to be catalytically active [162, 209]. With such hybrids, BMR was able to support high-level nitric oxide production by the fused nNOS heme domain, suggesting efficient electron transfer between the domains [209]. However, the protein stability of this hybrid enzyme was reduced and the rate of nitric oxide production was approximately eightfold lower than measured for native nNOS [209]. In contrast, with the converse hybrid, the nNOS reductase domain was rather unproductive at supporting reduction of the P450 BM3 heme domain, likely due to an inappropriate large distance between the flavin and heme redox centers [209]. Active fusions between RhFRed and a plant P450 has been recently described [210].

8.5 Substitution or Regeneration of NAD(P)H

The limited use of P450-catalyzed reactions in industry stems (at least to some extent) from the high cost of NAD(P)H cofactors. Consequently, several approaches have been developed and successfully applied to avoid the use of natural nicotine amide cofactors including chemical, electrochemical, and photochemical reduction of the heme Fe^{3+}. Another approach aiming to minimize the amount of NAD(P)H required comprises enzymatic cofactor regeneration. Moreover, several methods were described to directly convert P450s from their resting state into their active ferric hydroperoxy complex form, which enables substrate conversion without the need for cofactors or redox partners.

8.5.1 Chemical and Electrochemical Cofactor Substitution

8.5.1.1 Chemical Cofactor Substitution

At first sight, chemical reduction of ferrous iron appears to be a very simple and straightforward strategy to circumvent the use of costly NAD(P)H. Already in 1992, Peterson et al. demonstrated the effective reduction of ferrous iron by the strong and inexpensive reducing

agent sodium dithionite [211]. Later, the reductive capacity of sodium dithionite was shown to support P450 BM3-catalyzed hydroxylation of palmitic acid [212]. The hydroxylation reaction was carried out in two separate steps: anaerobic reduction and subsequent oxidation of P450 BM3 by oxygen bubbling. However, in both cases, the reduction rate of the heme iron was approximately 8000-fold slower than observed with NADPH [212]. Generally, strong reducing agents destabilize the porphyrin, which in turn results in low enzyme stability. This is probably one of the reasons why this approach has not been pursued.

Peroxides that directly convert the heme iron of P450s to a ferric hydroperoxy complex by the "peroxide shunt" (e.g., hydrogen peroxide, cumene peroxide, *tert*-butyl peroxide) might be useful for oxidation of various substrates. Some P450s are quite effective as peroxygenases, whereas others have to be engineered to become more efficient [213]. For example, CYP107A1 from *Streptomyces peucetius* was demonstrated to catalyze the H_2O_2-mediated dealkylation of 7-ethoxycoumarin [214]. CYP167A1 from *Sorangium cellulosum* was able to catalyze the oxidation of 7-ethoxy-4-trifluoromethylcoumarin when H_2O_2 was employed as the oxidizing agent [215]. Further, the group of Frances Arnold developed self-sufficient, peroxide-driven P450 BM3 catalysts [216, 217].

The essential problem in utilizing the "peroxide shunt" for P450 biocatalysis seems to lie in the time-dependent degradation of the heme and in oxidation of the protein [218, 219]. Therefore, methods of directed evolution, such as random and site-specific mutagenesis have been applied to evolve P450s to enhance the efficiency of the "peroxide shunt" pathway [216].

Natural P450 peroxygenases from the CYP152 family, such as CYP152B1 ($P450_{Spα}$) from *Sphingomonas paucimobilis* [220], CYP152A1 ($P450_{Bsβ}$) from *B. subtilis* [221], or CYP152A2 ($P450_{Cla}$) from the anaerobe *Clostridium acetobutylicum* [222], are attractive candidates for NAD(P)H-independent biocatalysis. However, the substrate spectrum of these P450s is restricted to fatty acids, which limits their practical applicability. It has been demonstrated, however, that the substrate spectrum of CYP152A1 can be extended by tricking its substrate recognition mechanism by the application of decoy molecules [144] (described in Sect. 8.3.2.3).

8.5.1.2 Electrochemical Cofactor Substitution

Electrochemical reduction of P450s seems to be a convenient way to supply electrons. Generally, such studies are typically performed to determine fundamental parameters of redox enzymes. Electrochemical reduction of P450s has been studied in detail for almost 20 years now and is sought-after for its potential use in biosensors or biocatalytic processes. The main idea behind these trials is to develop monooxygenases that could work in a "reactor plugged to a wall socket".

Electrochemistry of P450s has been investigated on graphite, glassy carbon, pyrolytic graphite, gold, platinum, or on metal oxide electrodes or nanostructured electrodes [223–225].

In brief, the main strategies of P450 electrochemistry are:

1. Indirect or mediated electron transfer utilizing redox compounds (so-called mediators) that are used to shuttle electrons between a P450 and an electrode; and
2. Direct electron transfer between an electrode and a P450.

The advantages and disadvantages of these strategies, as well as related examples, are discussed in detail in several reviews to which the interested reader is referred to [223–227].

For the indirect electrochemical regeneration of P450s, biological mediators (e.g., flavins) or electron carrier proteins (e.g., ferredoxins) are often applied. For example, electrochemical regeneration of P450cam was accomplished via cathodic reduction of Pdx [228–230]. Pdx was chosen as a natural redox mediator on account of the difficulty of transferring electrons directly from electrode to the interior heme of the large P450cam protein and in part because of the important role Pdx plays in maintaining the viability of the natural catalytic cycle (e.g., turnover rate, minimization of peroxide formation).

Another way to achieve indirect chemical reduction in solution is represented by the use of organometallic complexes (e.g., cobalt(III) sepulchrate trichloride). Often also redox partners and sometimes NAD(P)H are included in the reaction mixtures along with mediators [231, 232]. For example, application of a platinum wire working electrode supported the hydroxylation of lauric acid by recombinant CYP4A1 in the presence of rat CPR and cobalt(III) sepulchrate trichloride in solution. The product formation rate obtained was comparable to that obtained with NADPH [233]. In a similar system, P450 BM3 catalyzed the hydroxylation of lauric acid with a rate of 110 min^{-1} in the presence of cobalt(III) sepulchrate trichloride, whereas with NADPH, a hydroxylation rate of 900 min^{-1} was obtained [231].

One of the disadvantages of cobalt(III) sepulchrate trichloride is its aggregation. In addition, cobalt(III) sepulchrate trichloride can induce the production of reactive oxygen species in the system. The use of 1,1′-dicarboxycobaltocene as alternative mediator allowed to overcome the problem of mediator aggregation. In experiments with P450 BM3, 1,1′-dicarboxycobaltocene was observed to reduce the FAD and FMN in the reductase domain. The mediator was able to support lauric acid hydroxylation by the holoenzyme at a rate of 16.5 min^{-1}. Moreover, the heme iron in the separate monooxygenase domain could be reduced via 1,1′-dicarboxycobaltocene as well. The turnover rate in this case was 1.8 min^{-1} [234]. Nevertheless, the recognized limitations of this approach are low system efficiency and low sensitivity of mediators to molecular oxygen leading to high uncoupling. A possible strategy to minimize the uncoupling is the covalent attachment of the mediator to the enzyme resulting in a decreased distance between them.

For example, microsomal CYP2B4, CYP1A2, or mitochondrial CYP11A containing covalently bound riboflavin were immobilized on screen-printed rhodium–graphite electrodes and could be reduced [235]. Furthermore, the elegant concept of “molecular Lego” [150] (described in Sect. 8.3.3.1) to create artificial flavocytochromes has also been exploited for the generation of P450-based biosensors. Functional multidomain proteins with designed properties were generated beyond the restrictions imposed by the naturally occurring protein domains. For instance, the N-terminally modified human CYP3A4 was fused either to the reductase domain of P450 BM3 (BMR) or to the Fld from *D. vulgaris* and immobilized on modified glassy carbon or gold electrodes. The product formation and coupling efficiency of such systems were found to vary as a function of the electron transfer rate k_s’; the slowest k_s’ measured for CYP3A4–Fld fusion resulted in highest product formation and coupling. The authors explained the better performance for the slower k_s’ values through a longer-lived ferric–peroxy intermediate that leads to a better controlled catalysis [236].

The first direct electrochemistry for a P450 was reported in 1996 employing recombinant P450cam on an edge-plane graphite electrode [237]. Direct electrochemistry of P450s immobilized on a cathode is often complicated by a weak protein-mediated coupling between the heme and electrodes, because of the deeply buried prosthetic group or by unfavorable orientation of the protein on the electrode [238]. Moreover, the instability of enzymes upon interaction with the electrode surface represents a significant disadvantage of this method. Improvements include modifications of the electrode surface, e.g., by detergents [239] as well as entrapment of P450s in conductive polymers [240, 241], hydrophilic gels [242, 243], or biomembrane-like films [244–248].

The immobilization of P450 BM3 within didodecyldimethylammonium bromide (DDAB) films provided a very favorable environment for transferring electrons from the electrode to the heme iron. This transfer was measured directly and occurred at a fast rate (k_s’ = 221 s^{-1}), similar to the natural biological rate measured with palmitic acid as substrate. Furthermore, the electron transfer very much depended on the nature of the substrate and showed a lower k_s’ value of 130 s^{-1} when the less favored substrate lauric acid was used [223].

However, it has been demonstrated that high k_s’ values do not necessarily lead to catalytically active P450s [236]. Nevertheless, several

examples demonstrate the applicability of this approach for P450 biocatalysis. In situ entrapment of a P450 BM3 mutant in polypyrrole immobilized on platinum and glassy carbon electrodes resulted in a stable catalyst, which could be repeatedly applied in enzymatic reactions [249]. Thermostable CYP119 immobilized in a dimethyldidodecylammonium poly (*p*-styrene sulfonate) (DDAPSS) film has good retention of electrochemical activity up to 80 °C. Upon electrochemical reduction the CYP119–DDAPSS films demonstrated catalytic dehalogenation activities towards CCl_4, $CHCl_3$, and CH_2Cl_2 [250]. CYP1A2 or P450cam–polyion films grown layer-by-layer were employed on electrodes for catalytic oxidation of styrene derivatives to epoxides [246–248]. Further, the immobilization of microsomes on a polycation-coated electrode resulted in electrocatalytic oxidation of styrene [251].

One important finding from many studies on direct electrochemical heme reduction is that the heme redox couple is very sensitive to the presence of molecular oxygen (O_2), because oxygen is likely to be a strong competitor for electrons, thereby forming reactive oxygen species. For catalytic reactions involving P450s, the formation of reactive oxygen species, such as H_2O_2, is not desired because it dramatically reduces the efficiency of the catalytic process. Once generated, the ferrous heme rapidly binds dioxygen, but the catalytic reduction of O_2 to H_2O_2 usually follows quickly (Eqs. 8.2 and 8.3).

$$\mathbf{Fe^{2+} + O_2 \rightarrow Fe^{2+} - O_2} \tag{8.2}$$

$$\mathbf{Fe^{2+} - O_2 + 2H^+ + 2\bar{e} \rightarrow Fe^{2+} + H_2O_2} \tag{8.3}$$

The real challenge then, in any development of electrode-based biotransformations aimed at utilizing the P450 activities, is the use of the second electron for ferric peroxy complex formation rather than for H_2O_2 formation.

Generally, it seems that electrochemical approaches (at least at present) are not applicable for P450 biotransformations, but might be useful for the pharmaceutical industry for the investigation of drug-drug interactions, as well as for substrate screening in a biosensor arrangement [252–259].

8.5.2 Enzymatic Cofactor Regeneration

One of the most common approaches to overcome the stoichiometric need for NAD(P)H for P450 biotransformations involves application of an accessory enzyme for cofactor regeneration. Ideally, such enzymes need a sacrificial substrate that is cheap and innocuous. Moreover, both substrate and product of the cofactor-regenerating enzyme should be inert. Enzymatic cofactor regeneration is meanwhile a well-established approach applied for, e.g., alcohol dehydrogenases also at an industrial scale [260].

Common strategies for the enzymatic regeneration of NAD(P)H are based on D/L-isocitrate dehydrogenase (IDH), glycerol dehydrogenase (GlyDH), formate dehydrogenase (FDH), alcohol dehydrogenase (ADH), glucose dehydrogenase (GDH), or glucose-6-phosphate dehydrogenase (G-6P-DH) (Fig. 8.10).

P450 BM3 and its variant F87V were exploited for the preparation of (+)-leukotoxin B [(+)-12(*S*),13(*R*)-vernolic acid] from linoleic acid as well as 14(*S*),15(*R*)-epoxyeicosatrienoic acid from arachidonic acid, with application of G-6P-DH as the cofactor-regenerating enzyme (Fig. 8.11) [261].

Several cofactor regeneration systems were based on FDH. The substrate formate is an inexpensive, stable, and innocuous compound, while CO_2, which is produced by FDH, can be easily removed from the reaction by evaporation. A general drawback of FDH is, however, its low specific activity [262]. More stable FDH variants have been engineered and successfully applied [263]. Engineered FDH from *Pseudomonas sp.* 101, accepting not only NAD^+ but also $NADP^+$, has also been applied for NADPH regeneration [264]. For example, the maximal hydroxylation activity of P450 BM3 in solution towards the model substrate 10-*para*-nitrophenoxydecanoic acid was achieved by adding the engineered FDH

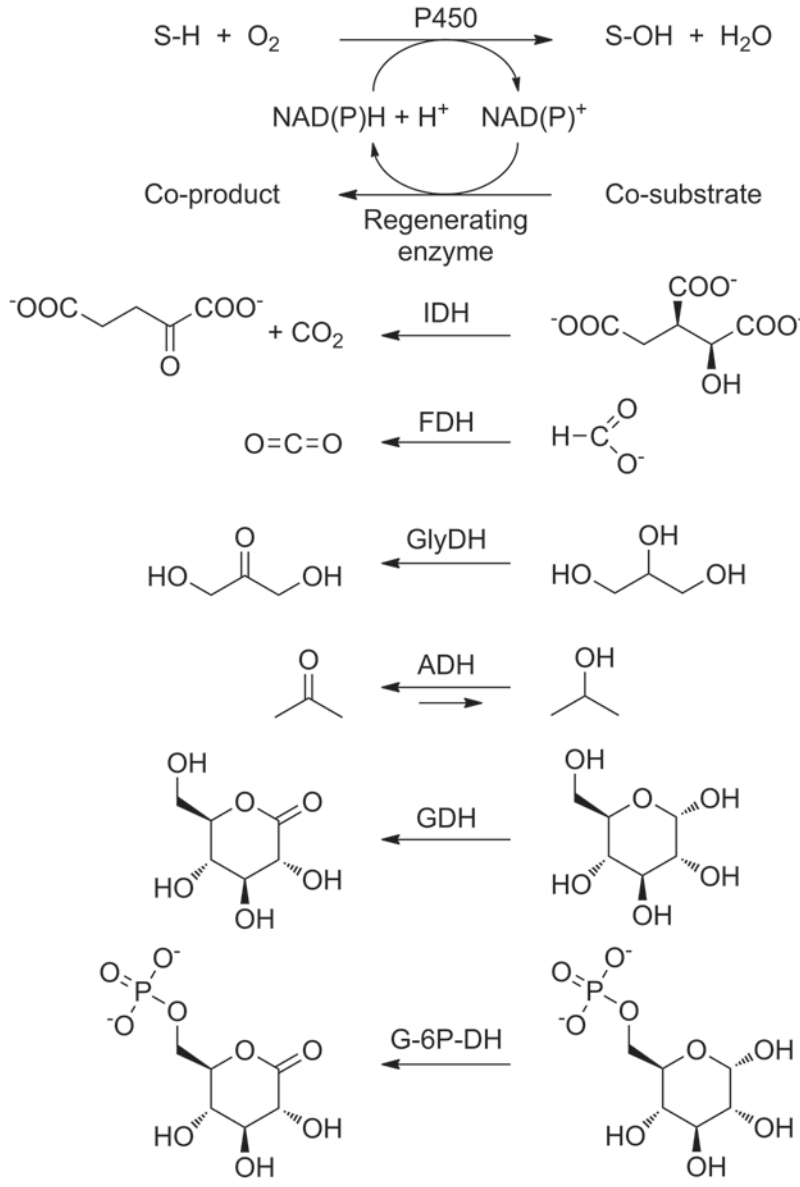

Fig. 8.10 Enzymes applied for regeneration of NAD(P)H in P450 biocatalysis. *IDH* D/L-isocitrate dehydrogenase, *FDH* formate dehydrogenase, *GlyDH* glycerol dehydrogenase, *ADH* alcohol dehydrogenase, *GDH* glucose dehydrogenase, *G-6P-DH* glucose-6-phosphate dehydrogenase

from *Pseudomonas sp.* 101. A tenfold excess of a P450 substrate over $NADP^+$ resulted in quantitative oxidation [265]. The same FDH variant supported P450 BM3-catalyzed reactions in biphasic systems with organic solvents [266, 267]. In such systems, the $NADP^+$-dependent formate dehydrogenase variant demonstrated a high operational stability under almost all tested reaction conditions.

A P450cam system with integrated enzymatic NADH regeneration by bacterial GlyDH was investigated in stable water-in-oil emulsions formed by the nonionic surfactant tetraethylene glycol dodecyl ether [268]. As a result, the cam-

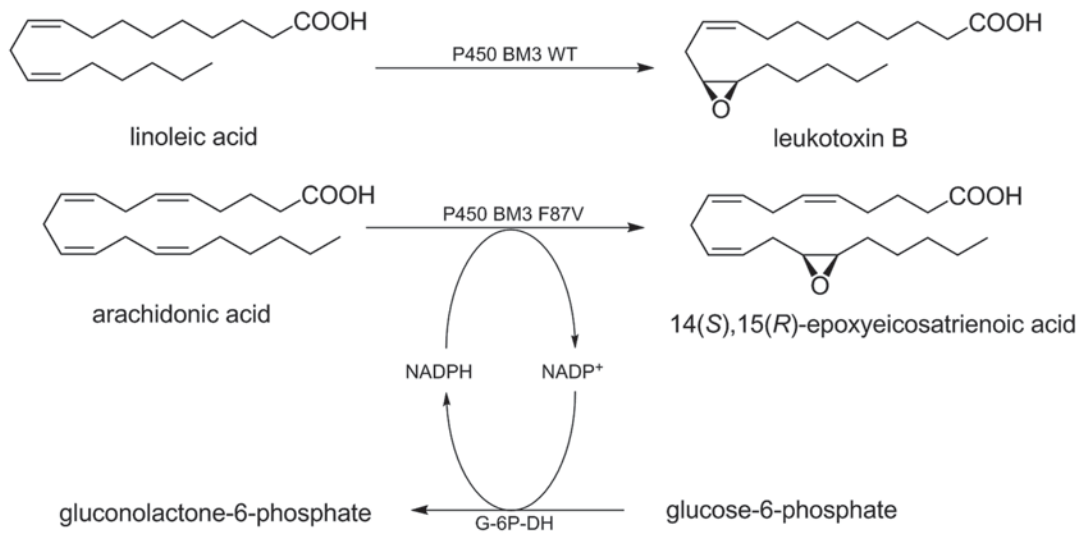

Fig. 8.11 Stereoselective synthesis of 14(*S*),15(*R*)-epoxyeicosatrienoic acid utilizing P450 BM3 F87V and glucose-6-phosphate dehydrogenase (G-6P-DH) for cofactor regeneration. Chemical steps yielded the corresponding antipode

phor hydroxylation rate was successfully improved approximately fivefold when GlyDH was employed [268].

A biocatalytic system containing P450 BM3 variants for the selective epoxidation of terminal alkenes and the commercially available alcohol dehydrogenase from *Thermoanaerobium brockii* for in vitro NADPH regeneration has been established [269]. In this case, the ADH was applied not only for cofactor regeneration but also because the alcoholic cosubstrate served as a cosolvent for the hydrophobic P450 BM3 substrates. A disadvantage of such systems, however, is that the substrate and product of a coupled ADH-catalyzed reaction are organic solvents (e.g., isopropanol and acetone), which might destabilize the P450 leading to lower productivities.

Some more complex systems have been tested for cofactor regeneration as well: P450 BM3 catalysis was linked to a two-step cofactor regeneration system composed of an NAD^+-dependent GlyDH and transhydrogenase from *E. coli*. Herein, P450 BM3 catalyzed the hydroxylation of a model substrate upon concomitant oxidation of NADPH to $NADP^+$, while simultaneously NADH was produced by GlyDH. Hydrides were subsequently transferred from NADH to $NADP^+$ by the transhydrogenase to form NADPH [270].

Recently, phosphite dehydrogenase (PTDH) from *Pseudomonas stutzeri* was applied to support cofactor regeneration for P450 BM3-catalyzed selective epoxidation of fatty acids, which was combined with a chemical metathesis [271]. PTDH and phosphite constitute a very promising system due to the great thermodynamic driving force for catalysis ($\Delta G^0 = -15$ kcal mol^{-1} compared to $\Delta G^0 = -5$ kcal mol^{-1} for FDH) and the low costs of the substrate phosphite [272]. During NADH regeneration with PTDH, a phosphite buffer was essentially converted to a phosphate buffer at a turnover rate of ~15,000 h^{-1} [273]. In addition, a PTDH variant has been generated that demonstrated high affinities to both NAD^+ and $NADP^+$ and thus can be used for the regeneration of both cofactors [274].

8.5.3 Photochemical (Light-Driven) Cofactor Regeneration

A number of photochemical approaches for cofactor substitution or regeneration to achieve ferrous heme reduction and support P450 catalysis have been reported. The main principle behind is the use of artificial photosensitive compounds, which mimic the function of photosynthetic or-

ganisms to convert light energy to chemical potential in the form of long-living charge-separated states. These processes are generally based on the photochemical reduction of flavins or other compounds mediating photo-induced electron transfer. In addition, the use of light-active cell components such as chloroplasts has been reported.

One report demonstrated the use of a non-covalently bound riboflavin for photo-induced intermolecular electron transfer from the isoalloxazine moiety of the flavin to the heme group of CYP2B4 [275]. Although an effect of different substrates on the electron transfer rate in this artificial system was observed, no product formation was reported.

We recently reported the use of a light-driven approach based on photo-excited flavins and the electron donor ethylenediaminetetraacetate (EDTA) as the electron source for in situ generation of H_2O_2 to support the CYP152 peroxygenases $P450_{Bs\beta}$ and $P450_{Cla}$ [276]. The peroxygenase activities determined for these systems were generally lower than those observed after direct addition of H_2O_2 (since they strongly depend on the ratio of H_2O_2 to P450). However, the in situ generation of H_2O_2 proved to be advantageous, since these systems generally displayed a better operational stability and therefore allowed higher overall substrate conversions.

An unconventional application of the ruthenium *tris*(2,2′-bipyridine)-linked heme group of myoglobin has been reported by two research groups [277, 278]. Herein, oxidation of ferric heme iron was performed by photo-activated Ru(bpy)3 resulting in compound I species ($Fe^{IV}=O$). This strategy was successfully extended to P450 BM3 [279]: Covalent linkage of a Ru^{II}-diimine photosensitizer to a cysteine near the heme group promoted electron transfer from the heme Fe^{III} to photogenerated Ru^{III}. Flash-quench oxidation of the ferric-aquo heme yielded the Fe^{IV}-hydroxide species (compound II). Finally, several hybrid P450 BM3 heme domains containing a covalently attached Ru^{II} photosensitizer at different cysteines near the heme groups, as well as substitution of two other cysteines, have been constructed and studied with respect to stability, labeling properties, and catalytic activity towards lauric acid. The best hybrid Ru^{II}–L407C–Fe^{III} demonstrated the highest stability and catalyzed the light-driven hydroxylation of lauric acid with total turnover numbers of 935 and an initial reaction rate of 125 $nmol_{product}$ min^{-1} $nmol_{P450}{}^{-1}$ [280].

A promising resourceful approach represents the rerouting of natural photosynthetic electron transfer into the biosynthetic production of high-value products by P450s. Upon irradiation, the natural photosystem II in chloroplasts splits a water molecule, thereby generating molecular oxygen, whereas photosystem I transfers electrons to $NADP^+$, yielding NADPH. This NADPH can be then applied for P450-catalyzed reactions in artificial systems. Already in earlier studies, attempts have been made to use plant chloroplasts for the development of light-driven P450 systems. A light-driven P450 catalysis has been performed by mixing isolated spinach chloroplasts and yeast microsomes containing a genetically engineered fusion of rat CYP1A1 and yeast CPR [281]. Upon irradiation, this mixture supported conversion of 7-ethoxycoumarin to 7-hydroxycoumarin. The same system was immobilized by different methods to prove its applicability for biocatalytic processes [282]. Herein, entrapment in agarose resulted in the highest conversion. A two-phase column-type reactor with separately immobilized microsomes and chloroplasts performed best and exhibited a higher conversion as compared to a reactor with coimmobilized components, with turnover rates of 6.3 and 2.5 $nmol_{product}$ min^{-1} $nmol_{P450}{}^{-1}$ after 40 and 180 min, respectively.

Recently, in a similar study, the isolated photosystem I from barley (*Hordeum vulgare*) was combined with spinach Fdx and the membrane-bound CYP79A1 from Sorghum (*Sorghum bicolor*) [283]. Upon irradiation, CYP79A1 catalyzed hydroxylation of L-tyrosine to oxime. In addition to spinach Fdx, also Fld from a photosynthetic cyanobacterium *Synechococcus* sp. was able to support transfer of electrons from NADPH to CYP79A1, thereby enabling catalysis, but at a much lower rate [283]. Recent trials to replace NADPH production via regeneration systems by

light and electron transfer via the photosynthetic system from barley have been also reported for bacterial soluble CYP124 from *Mycobacterium tuberculosis* [284]. The first results of light-driven P450 biocatalysis seem to be very promising, but further studies are necessary to compare the efficiency and sustainability of such systems with those using recombinant microorganisms [285].

8.6 Whole-Cell Processes with P450 Enzymes

Due to the cofactor dependency and the multicomponent nature of P450 systems, as well as the need for membrane integration in the case of eukaryotic P450s, their industrial applications have so far been restricted to whole-cell systems, which take advantage of the host's endogenous cofactor regeneration systems (and sometimes also its redox partners). In such instances, however, physiological effects like limited substrate uptake and product efflux by the microbial cell, toxicity of substrate or product, product degradation, and elaborate downstream processing have to be taken into account (see Sect. 8.1.2) [34, 286]. Moreover, when titers of a recombinant P450 biocatalyst within the cell reach a certain threshold, the cofactor concentration may again become a bottleneck for the overall process.

8.6.1 Production of Drug Metabolites

With respect to their biotechnological potential, P450s play a vital role in the field of drug transformation. They are important enzymes in phase I drug metabolism reactions in humans and are responsible for the initial oxidation of xenobiotics. Out of the 57 P450 isoenzymes that are expressed in human, focus is given to CYP1A2, 2C9, 2C19, 2D6, and 3A4 since they mediate about 75–80% of the drug metabolism [287, 288]. Detailed investigation of the properties of drug metabolites is an essential prerequisite for the assessment of drug-induced side effects, drug–drug interaction, and drug toxicity. Since drug metabolite standards are in most cases not commercially available or difficult to synthesize by chemical means, P450s are the most important enzymes for the biotransformation of drugs and the preparation of metabolites.

While bacterial P450s are mainly soluble enzymes that can be expressed in high amounts in bacterial expression systems, eukaryotic P450s are membrane-bound enzymes, which render their expression much more difficult. Therefore, it is not surprising that many attempts have been focused on engineering eukaryotic P450s for successful expression in recombinant hosts [104, 289]. In most cases, *E. coli* was selected as the appropriate host system because of its easy handling, inexpensive culture medium, and rapid growth. It is widely recognized that membrane-bound regions of eukaryotic P450s can severely reduce the yield of heterologous protein expression in prokaryotic hosts [290, 291]. Therefore, most of the work aimed at tailoring membrane-bound P450 enzymes for soluble expression in *E. coli* concerned modifications of the hydrophobic N-terminal amino acid sequence. The main strategies include mutagenesis of this region [292, 293], replacement by an optimized N-terminal sequence of bovine CYP17A1 [29], complete or partial removal of the N-terminal sequence [294–298], or a combination of these approaches. Furthermore, the introduction of the signal peptide from OmpA or PelB at the N-terminus of several microsomal P450 improved the integration into the bacterial inner membrane [299]. Also modifications within the F–G loop [300] as well as protein engineering performed on the whole gene [289, 301, 302] led to significantly enhanced concentrations of the recombinantly expressed mammalian P450s. There is, however, no possibility to predict the effect of such modifications on the expression level beforehand, and successful expression is not guaranteed.

The application of recombinant human P450s for the production of drug metabolites is by now widely established [303]. Pharmaceutical companies (e.g., Novartis Pharma AG, Hoffmann-La Roche, or Codexis) have implemented collections of recombinant human CYP isoenzymes, which have a number of advantages over hepatic microsomes or recombinant insect cells

[304, 305]. For example, Novartis Pharma AG has created *E. coli* strains in which 14 different recombinant human CYPs are functionally co-expressed with human CPR [303]. Recombinant *E. coli* strains expressing various human P450s can be cultivated at scales of up to 100 L [303, 306]. Importantly, up to 300 mg of different drug metabolites could be obtained by application of permeabilized resting *E. coli* whole-cells at a 1–2-L-scale production [306]. The enzymes are used as biocatalysts for the biosynthesis of drug metabolites as well as for *d*rug *m*etabolism and *p*harmaco*k*inetics (DMPK) applications, for example, in P450 inhibition screenings [306].

Recombinant human P450s have also been expressed in eukaryotic expression hosts, which further facilitated their use for the synthesis of drug metabolites. A recent overview describing various recombinant systems including bacteria, yeast, and mammalian cell cultures is provided in [307]. For example, several microsomal human P450 isoforms have been coexpressed in fission yeast *Schizosaccharomyces pombe* together with either human CPR or with its homologues from fission yeast (ccr1) or the bishop's weed *Ammi majus* (AmCPR). In total, 28 recombinant strains were constructed and compared regarding their synthetic efficiency towards several drugs. P450 activities were shown to differ depending on the P450-CPR combination: While CYP3A4 was more active with human CPR, CYP2D6 displayed its highest activity when coexpressed with ccr1, whereas CYP2C9 showed highest activity with AmCPR [308].

Besides recombinant human P450s, microbial wildtype strains that are natural producers of the compound of interest, as well as recombinant strains expressing bacterial P450s, have been shown to be eligible by research institutions and pharmaceutical companies for the larger-scale (100 mg to multi-g) synthesis of drug metabolites. Microbial strains, as well as recombinant and engineered P450s, are of particular interest for the identification and production of nonhuman metabolites with new biological activities. Pharmaceutical companies possess collections of bacteria, yeasts, and fungi to systematically screen target drugs with the goal to identify additional P450s with new substrate ranges. Interesting candidates have been identified in among others in the genera *Cunninghamella, Curvularia, Aspergillus, Rhizopus, and Streptomyces* [309].

A number of studies have been dedicated to protein engineering of bacterial P450s for the production of drug metabolites [310]. A vast number of reports describe mutants of P450 BM3. The first evidence that P450 BM3 can bind drug-like molecules was provided in 2005 by Nico Vermeulen and coworkers [311]. One year later, the triple mutant R47L/F87V/L188Q was found to metabolize testosterone, amodiaquine, dextromethorphan, acetaminophen, and 3,4-methylenedioxymethylamphetamine [122]. Consequently, P450 BM3 has been extensively engineered to metabolize various drugs by using site-directed mutagenesis, site-saturation mutagenesis, directed evolution, or a combination of these approaches [123, 143, 310, 312–315]. Frances Arnold and coworkers have created a library of CYP102A-chimeras demonstrating completely new activities including the ability to metabolize a number of drugs [136, 312]. Importantly, several products were formed with high regioselectivity.

It was shown that also other bacterial and fungal P450s can be applied for the production of drug metabolites. For example, wild-type CYP105A1 was able to produce human drug metabolites from glimepiride and glibenclamide [316]. Also a fungal self-sufficient P450 from *Aspergillus fumigatus* expressed in *E. coli* was successfully applied to produce the human metabolites 4′-hydroxy-diclofenac and 6-hydroxychlorzoxazone [317]. Taken together, investigations of microbial recombinant P450s may provide a large reservoir of enzymes for the production of drug metabolites with very different structures.

8.6.2 Production of Building Blocks for Chemical Synthesis

P450s are widely used not only for the production of drug metabolites but also in the synthesis of drug compounds or their precursors. Among

these are steroid-based compounds, which are widely used as "anti-agents," exhibiting, for example, antitumor, anti-inflammatory, antimicrobial, antiviral, antifungal, or antiallergic functions [318]. On a molecular level, steroid hormones are known to be involved in cell proliferation and tissue differentiation, in regulation of signal transduction and in other vital processes [319–321]. Probably, the best-established commercial applications of natural strains are the 11α-hydroxylation of progesterone to yield cortisone by *Rhizopus* sp. (former Pharmacia & Upjohn, now Pfizer Inc.) [322, 323] and the 11β-hydroxylation of 11-deoxycortisol to cortisol with *Curvularia* sp. established at an industrial scale of approximately 100 t/year (former Schering AG, now Bayer HealthCare Pharmaceuticals) [324]. Both processes involve one or two oxidation steps catalyzed by fungi starting with complex precursor steroid molecules, such as diosgenin, which are isolated from plants and subsequently chemically derivatized [325, 326]. High productivities and low production costs of the oxidized steroid products have been reached by optimizing the production strains and establishing high cell density fermentations of stable biocatalysts. However, detailed information on the production conditions has not been released.

Also in the field of steroid transformations, a number of successful developments in genetic and metabolic engineering and whole-cell biocatalysis have been reported recently. The physiological activity of steroids depends on their structure as well as on the number and stereo- and regio-position of the functional groups on the steroid core. It is obvious that steroid hydroxylases with different stereo- and regioselectivities are needed. One interesting example is provided by the screening of a recombinant library containing 250 bacterial wildtype P450s expressed in *E. coli* for testosterone oxidation. This screening identified 24 bacterial P450s that monohydroxylate testosterone in a regio- and stereoselective manner at the 2α-, 2β-, 6β-, 7β-, 11β-, 12β-, 15β-, 16α-, or 17-positions [327, 328]. Most of these hydroxylations are common for both prokaryotic and human P450s. Therefore, the identified bacterial candidates can be applied without further modifications for the production of human drug metabolites on a preparative scale. The majority of these bacterial P450s originate from actinomycetes, which have been studied extensively in the last years with respect to steroid degradation [329, 330]. Therefore, it is not surprising that two novel steroid hydroxylases, which were recently characterized, originate from actinomycetes [331, 332]. Both, CYP154C5 from *Nocardia farcinica* IFM10152 [331] and CYP154C3 from *Streptomyces griseus* [332] demonstrated high regio- and stereoselectivity for the 16α-position and produced 16α-hydroxylated derivatives of steroids like testosterone, pregnenolone, and progesterone.

The regioselectivity of steroid hydroxylases can successfully be improved or altered by the means of protein engineering as was demonstrated for the 15β-steroid-hydroxylase CYP106A2 from *B. megaterium,* which was engineered to produce 11α-hydroxyprogesterone [138, 139]. In another study, human CYP2D6 was mutated at two active site positions with the aim of constructing a regioselective steroid hydroxylase [333]. Four hundred possible combinatorial mutations at these two positions were generated and the corresponding mutant P450s were expressed individually in *Pichia pastoris* and tested for activity with testosterone as a model substrate. High-performance liquid chromatography-tandem mass spectrometry (HPLC-MS) analysis revealed several CYP2D6 mutants with improved activity and selectivity towards the 2β-position, which is not oxidized by the wildtype enzyme.

Apart from the usually low activity and sometimes insufficient selectivity of P450s towards steroids (which can be improved by means of protein engineering), the low solubility of steroid compounds in water (1–100 μM [334]) represents a challenging problem for the establishment of whole-cell biocatalysis. Consequently, several promising reaction-engineering techniques that were applied for biotransformations of other hydrophobic compounds have also been tested with steroid substrates. Among these are (1) biphasic reaction setups with an organic phase, which serves as substrate reservoir, (2) surfactant-

facilitated emulsification of steroids, and (3) the use of solubilizing agents such as cyclodextrins [335, 336]. In addition, biphasic systems or the addition of resins can partially solve the problem of product degradation by in situ product recovery [337].

Low substrate uptake into the microbial cell might hinder effective whole-cell biotransformation as well. In these cases, exchange of the expression host can sometimes improve the biocatalyst performance significantly. Recently, a xylose-inducible expression system based on *Bacillus megaterium* MS941was constructed, in which CYP106A2 was coexpressed with the heterologous redox partners AdR and Adx. It was demonstrated that the pentacyclic triterpene 11-keto-boswellic acid can efficiently cross the membrane of the recombinant *B. megaterium* cells, while it did not occur when using recombinant *E. coli* cells, where no activity was observed [89]. The authors suggested that this is probably due to the inability of hydrophobic acids to cross the outer membrane of the *E. coli* cells. The optimized whole-cell *B. megaterium* biocatalyst achieved a space–time yield of 561 mg 15α-hydroxylated 11-keto-β-boswellic acid L^{-1} day^{-1} with 80% product selectivity [89].

The alkane-assimilating yeast *Yarrowia lipolytica* that possess an efficient uptake system for hydrophobic substances was used as expression host for human CYP2D6 and CYP3A4 along with human CPR. Recombinant *Y. lipolytica* was successfully used for the conversion of poorly soluble steroids like testosterone and progesterone in a biphasic system with ethyl oleate [338].

On the other hand, treatment of *E. coli* with the peptide antibioticpolymyxin B led to an effective permeabilization of *E. coli* supporting the entry of abietic acid, which led to an almost fivefold improved conversion of abietic acid into 15-hydroxyabietic acid by CYP105A1 [92].

The limited uptake of the precursor cholesterol into the cells in general and the need for sustainable steroid production based on renewable resources fuelled the development of the industrially relevant de novo artificial biosynthesis of hydrocortisone starting from endogenous ergosterol in recombinant *Saccharomyces cerevisiae* [339, 340]. The biosynthesis of ergosterol, which is the major yeast sterol, was rerouted by cloning and expression of a Δ7-reductase from *Arabidopsis thaliana* to produce precursors resembling cholesterol, namely ergosta-5-ene-ol and ergosta-5,22-diene-ol, which in turn served as substrates for bovine CYP11A1. The genes encoding the redox partners for CYP11A1, i.e., AdR and Adx, were coexpressed in the engineered yeast as well. With such a system, a total pregnenolone concentration of 60 mg L^{-1} was obtained. By additional coexpression of human 3β-hydroxysteroid dehydrogenase/isomerase, pregnenolone could further be converted to progesterone [339]. Subsequent conversion of progesterone to hydrocortisone via the intermediates 17-hydroxy-progesterone and 11-deoxycortisol was catalyzed by the heterologously expressed CYP17A1, CYP21B1, and CYP11B1. Thus, an artificial biosynthetic pathway for the production of hydrocortisone was established in a single yeast strain by expressing nine engineered recombinant mammalian and plant genes [340].

Another well-documented industrial process is the production of pravastatin via 6β-hydroxylation of the precursor compactin (also referred to as mevastatin) by *Streptomyces carbophilus* (Daiichi Sankyo and Bristol-Myers Squibb) [341–343]. Statins inhibit 3-hydroxy-3-methyl-glutarylcoenzyme A (HMG-CoA) reductase, which is involved in cholesterol biosynthesis [344]. The biotechnological production of pravastatin consists of two steps: compactin is first produced in *Penicillium citrinum* and then hydroxylated at position 6β to form the target product pravastatin by *S. carbophilus* [345]. While *S. carbophilus* has been successfully used for industrial production of pravastatin, further investigations on this system were undertaken for its improvement. *S. carbophilus* is sensitive to compactin, which inhibits cell growth and causes cell lysis, which in turn limits the production of pravastatin [345, 346]. Hence, a search for less sensitive and more effective biocatalysts via microbial screening has been and still remains one of the main foci of process optimization [347, 348]. Moreover, significant advances were made with respect to the identification and mutagenesis of CYP105A3 in *S. carbophilus* (known as P450 sca-2), which was demonstrated to be responsible

for the stereo- and regioselective hydroxylation of compactin [165, 342]. Recently, an artificial redox chain consisting of CYP105A3, together with PdR and Pdx from *P. putida*, was constructed and optimized by means of protein engineering, resulting in mutants that exhibited a more than tenfold increased activity [349]. Another improvement in pravastatin production could be achieved by implementation of recombinant *E. coli* whole-cell systems expressing CYP105A3 with disrupted AcrAB and TolC efflux pump systems resulting in a higher biocatalytic efficiency [350]. The strongest effect was achieved after the disruption of TolC, which led to a sevenfold increased pravastatin level. The author suggested that the positive effect is due to the reduced compactin efflux out of the cell [350].

8.6.3 Optimization of Whole-Cell Biocatalysts

Low substrate solubility as well as strain-related physiological limitations, as discussed above, have been addressed in many independent studies that focused on whole-cell based P450 transformation of a variety of hydrophobic compounds. It has been demonstrated for medium- and long-chain aliphatic compounds that low substrate transfer rates across the membrane into *E. coli* cells is one of the major limiting steps in whole-cell biotransformations [351, 352]. To overcome inefficient uptake of pentadecanoic acid by intact *E. coli* cells harboring fatty acid hydroxylase P450 BM3, the *alkL* gene belonging to the alkane uptake system of *P. putida* GPo1 (formerly known as *Pseudomonas oleovorans*) was cloned and coexpressed, which led to an at least twofold increased hydroxylation rate [352]. In addition, improved substrate uptake in recombinant *E. coli* has recently been confirmed for dodecanoic acid methyl ester as substrate and attributed to the function of AlkL as outer membrane transporter [353]. This strategy was successful with other P450s. For instance, coexpression of AlkL resulted in a fivefold enhanced (*S*)-limonene oxidation catalyzed by recombinant *E. coli* expressing CYP153A6 [354] or led to improved production of ω-hydroxy dodecanoic acid (4 vs. 1.2 g L^{-1}) in a biphasic system using recombinant *E. coli* cells expressing the artificial fusion CYP153A–BMR (reductase domain of P450 BM3) as biocatalyst [355]. Alternatively, in order to circumvent the sensitivity of *E. coli* to organic solvents, solvent-resistant strains like *P. putida S12* [356] or *B. subtilis* 3C5N can be used as alternative production hosts [357].

Substrate or product toxicity can seriously affect biotechnological application of P450s, as was demonstrated, e.g., for *S. cerevisiae* producing the sesquiterpenoid fragrances β-nootkatol and nootkatone by heterologously expressed plant CYP71D51v2. Both products were toxic for yeast at concentrations exceeding 100 mg L^{-1}, which hampered the application of this system for the industrial bioconversion of valencene [358]. A recombinant *E. coli* containing bacterial CYP109B1 was shown to produce nootkatol and nootkatone at up to 120 mg L^{-1} in a biphasic system, without a significant effect on bacterial performance [359].

As long as living cells are provided with the necessary nutrients, all endogenous cofactor-recycling systems are functional and there is no need to supplement cells with external nicotine amide cofactors. However, when the activity of a recombinant P450 or its concentration in the cell reaches a certain threshold, or in cases where the uncoupling between NAD(P)H consumption and product formation is high, the concentration of cellular NAD(P)H can become limiting for P450 catalysis [360]. In such cases, cofactor-regenerating enzymes coexpressed together with target P450s can help to improve the biocatalytic process.

When GlyDH was coexpressed together with P450cam and its physiological redox partners in *E. coli,* a tenfold higher camphor conversion was observed compared to a system without GlyDH (37 vs. 4%, respectively). Notably, conversion was performed without the addition of glycerol to the reaction mixture, which indicated that endogenous glycerol was efficiently utilized by GlyDH. In an aqueous system with ethanol as cosolvent, a camphor conversion of 100% was achieved after the addition of 10% (v/v) glycerol to the reaction mixture [361].

Another approach in this field is the construction of an *E. coli* whole-cell biocatalyst with

improved intracellular cofactor regeneration driven by external glucose [362]. In this system, additional intracellular NADPH regeneration occurs through coexpression of a glucose facilitator from *Zymomonas mobilis* for the uptake of non-phosphorylated glucose and a $NADP^+$-dependent glucose dehydrogenase from *B. megaterium,* which oxidizes glucose to gluconolactone. This strain was successfully utilized for the oxidation of the cyclic monoterpene α-pinene catalyzed by a mutant of P450 BM3 and showed a nine times higher initial α-pinene oxide formation rate and a sevenfold increased α-pinene oxide yield in the presence of glucose as compared to glucose-free conditions [363].

In a different system, the heterologous proteins Adx, AdR, and CYP106A2 were coexpressed in *E. coli* along with an alcohol dehydrogenase from *Lactobacillus brevis* [364]. This whole cell biocatalyst was then applied for the oxidation of progesterone and testosterone to the corresponding 15β-hydroxylated derivatives. 2-Propanol was chosen as solvent for the steroids and as a substrate for the alcohol dehydrogenase. The highest activity was observed in the presence of 2 M 2-propanol (15.4% v/v), which was suggested to be largely due to enhanced substrate solubilization rather than improved intracellular cofactor regeneration. In order to overcome the problem of impaired substrate transport across the cell membrane, lyophilized cell free extracts were applied for this system, which increased the productivity up to 18-fold as compared to the *E. coli* whole-cell catalyst without cofactor regeneration [364].

8.7 Microbial de novo Synthesis of Plant Secondary Metabolites and Transgenic Plants

8.7.1 Microbial Synthesis of Plant Secondary Metabolites Using P450s

For long, plant secondary metabolites have been utilized by mankind; they are relevant to health and nutrition issues and are still a main source for new pharmaceuticals [365, 366]. However, usually only small amounts can be obtained from plants, due to slow plant growth and low concentrations of the secondary metabolites in plant material. Other drawbacks may arise from season-dependent variations in secondary metabolite yields and high phytochemical background in plants producing many similar substances [367–370].

To satisfy the growing demand for scalable production of plant metabolite-based pharmaceutical drugs, alternative production strategies are necessary. Chemical de novo syntheses of these structural complex substances are not trivial and normally include many waste-generating reactions and purification steps, causing high expenses and resulting in low product yields [371]. Microbial production systems on the other hand represent a promising alternative. Such systems exhibit high growth rates and allow the use of renewable resources, which offers short production periods and limits waste accumulation.

While many efforts have been made to engineer microbes for the production of secondary metabolite core structures, the implementation of the tailoring steps to diversify highly functionalized molecules still remains a challenge. The high number of genes encoding P450s identified in the genomes of plants reflects the important role of these enzymes in (secondary) metabolic pathways [372]. However, the function of many of these P450s is still unknown, explaining why only early steps or partial plant secondary metabolite pathways have been implemented in microbes so far.

Metabolites generated in microbes often serve as a starting point for chemical routes towards the target products, but are also necessary for the functional testing of candidate enzymes to further elucidate metabolic pathways [373, 374]. Besides the biosynthetic routes starting from renewable feed stocks such as sugars or glycerol, also commercially available intermediates might be used in cases when information on early pathway steps is lacking [375].

For the implementation of the secondary metabolite pathways involving P450s, a suitable microbial host is required. The selection criteria

for a suitable host usually involve product/substrate tolerance, intrinsic availability of precursors, genetic accessibility, and ability to grow to high cell densities [376]. The model organisms *E. coli* and *S. cerevisiae* have been successfully employed for secondary metabolite production. The overwhelming majority of plant P450s, as well as their dedicated redox partner CPRs, are localized in the endoplasmic reticulum (ER). For the heterologous expression of plant P450s, *S. cerevisiae* with its ER membrane, native P450s, and CPR seems advantageous as compared to *E. coli,* which lacks an ER and does not possess any P450. Nevertheless, P450s and CPRs have also been successfully expressed in *E. coli,* mainly by engineering of the transmembrane regions of these enzymes [377].

The next section aims to point out the potential and limitations of P450s as part of pathways for the production of plant secondary metabolites in *E. coli* and *S. cerevisiae*.

8.7.1.1 De novo Synthesis of Terpenoids

With more than 60,000 isolated substances (Dictionary of Natural Products; http://dnp.chemnetbase.com; 2014/03/27), terpenoids represent the structurally most diverse group of plant secondary metabolites. Often several P450s are involved in the biosynthesis of these highly oxidized compounds [71, 378]. Terpenoids are considered high potential pharmaceuticals and thus many attempts have been undertaken to engineer microbes for terpenoid production. Such engineering approaches usually follow a similar scheme: Either the mevalonate (MVA) or the methylerythritol phosphate (MEP; also designated as Non-MVA or DXP) pathway is used to generate the universal isoprenoid precursors isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) [379–386]. These are further converted to the respective terpenes by prenyltransferases and terpene cyclases. As a last step, tailoring enzymes such as P450s are integrated to produce the desired terpenoids. In some cases, only a single P450 is necessary to catalyze several steps, yielding the respective terpenoic acids.

8.7.1.1.1 Monoterpenoids

An early attempt for the de novo biosynthesis of a plant secondary metabolite, including a P450-catalyzed step, was aimed at the production (−)-carvone [387]. Carvone is the main component of spearmint (*Mentha spicata*) essential oil. Interestingly, in addition to its use as a fragrance and flavor additive, carvone exhibits antimicrobial and cancer chemopreventive activity. To achieve (−)-carvone production, the native MEP pathway of *E. coli* was exploited and geranyl diphosphate synthase, limonene synthase, an artificial CYP71D18-CPR fusion, and carveol dehydrogenase were simultaneously expressed in *E. coli* (Fig. 8.12). Although 5 mg/mL limonene could be produced, significant accumulation of (−)-carvone occurred only when external limonene was fed. The amounts of (−)-carvone, however, never exceeded 2 μM (ca. 0.3 mg/mL), which was attributed to the suboptimal expression of the P450–CPR fusion protein and low intracellular concentration of limonene caused by the poor solubility and uptake as well as efficient excretion by the host.

Recently, the production of the antitumor agent perillyl alcohol was established in *E. coli* [388]. This secondary metabolite is usually produced by *Perilla frutescens* via limonene-7-hydroxylase (CYP71D174)-mediated hydroxylation of limonene with 50 % specificity [389]. By implementation of the nonnative MVA pathway, as well as the introduction of geranyl diphosphate synthase and limonene synthase, 400 mg L^{-1} limonene could be produced in *E. coli* (Fig. 8.12). Bacterial CYP153A6 and its redox partners were additionally introduced in *E. coli* for the further hydroxylation. Although CYP153A6 shows a high specificity as compared to CYP71D174 and, in contrast to many plant P450s, was shown to be expressed at high levels in *E. coli,* only 100 mg L^{-1} perillyl alcohol could be produced. It thus appears that the P450-catalyzed reaction is a bottleneck in the production of perillyl alcohol. An additional contributing factor likely is the extreme toxicity of monoterpenes towards microbial organisms. The latter phenomenon was partially overcome by the use of resins for in situ product removal [388].

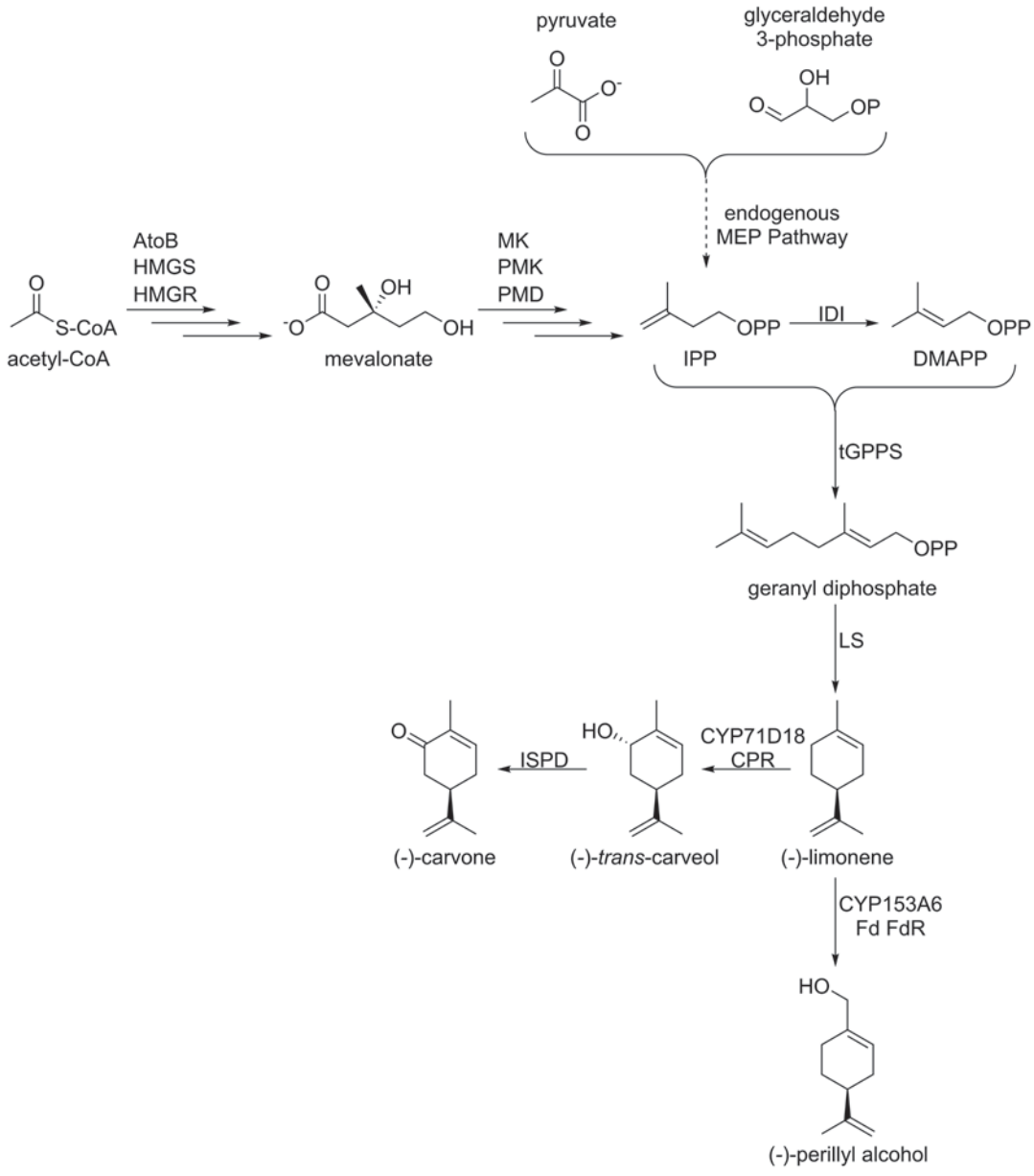

Fig. 8.12 Heterologous and endogenous pathways used for the production of the monoterpenes (−)-carvone and (−)-perillyl alcohol in *E. coli*. *AtoB* acetyl-CoA acetyltransferase *HMGS* hydroxymethylglutaryl-CoA synthase, *HMGR* 3-hydroxy-3-methylglutaryl-coenzyme A reductase, *MK* mevalonate kinase, *PMK* phosphomevalonate kinase, *PMD* diphosphomevalonate kinase, *IDI* isopentenyl-diphosphate isomerase, *tGPPS* truncated geranyl diphosphate synthase, *LS* limonene synthase, *ISPD* (−)-isopiperitenol/(−)-carveol dehydrogenase

8.7.1.1.2 Sesquiterpenoids

Several sesquiterpenoids have already been produced in microorganisms (Fig. 8.13). The least complex example is the production of the grapefruit aroma compounds nootkatol and nootkatone in yeast, relying on farnesyl diphosphate (FPP) supplied via the endogenous MVA pathway in studies aimed at the identification of valencene

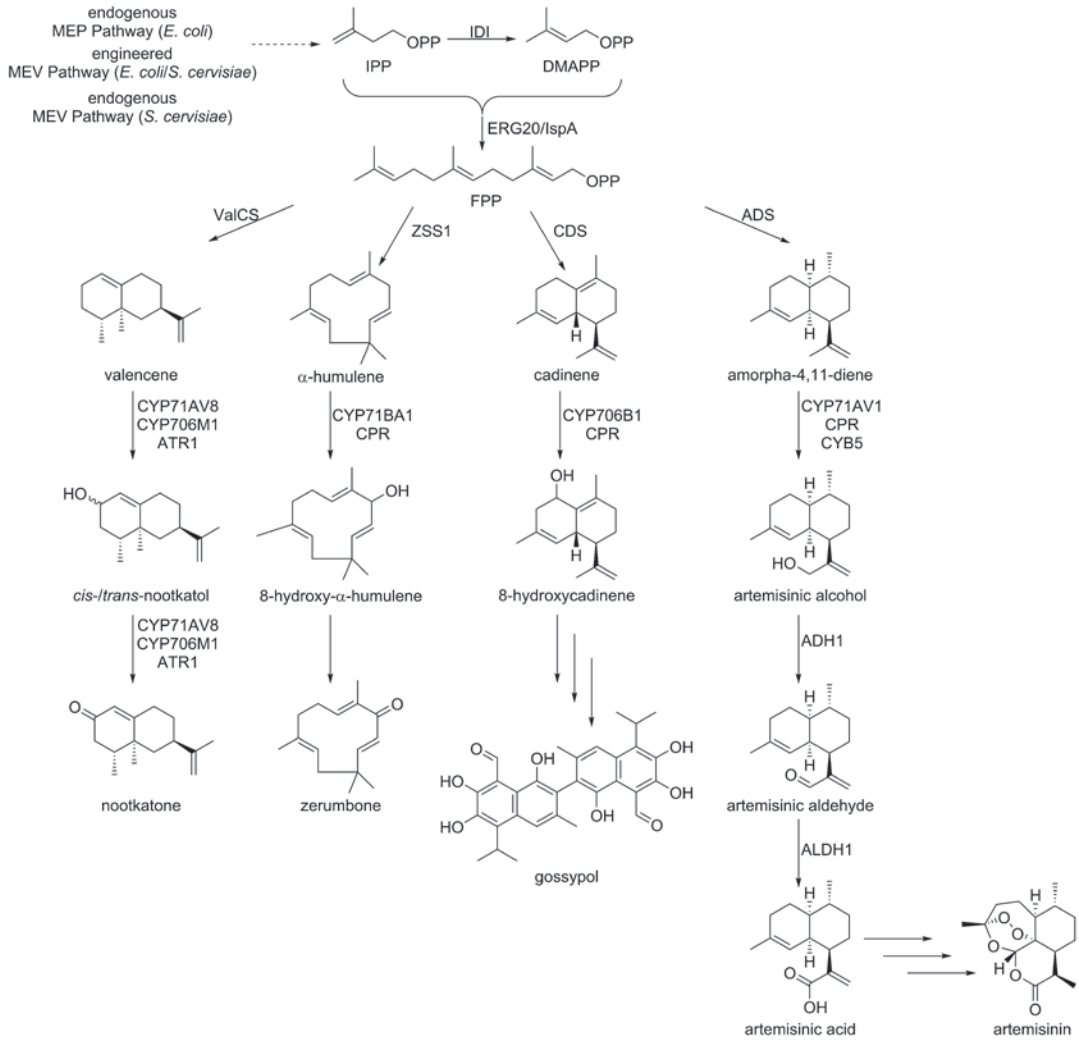

Fig. 8.13 Pathways employed for the production of nootkatone, 8-hydroxy-α-humulene, 8-hydroxycadinene, and artemisinic acid in microbial hosts. Further downstream metabolites like zerumbone, gossypol, and artemisinin are also shown. In studies using *E. coli,* an engineered mevalonate (*MVA*) pathway as well as the endogenous methylerythritol phosphate (*MEP*) pathway and farnesyl diphosphate (*FPP*) synthase support FPP production. When using *S. cerevisiae* as host, either the native (for nootkatone production) or an optimized homologous MVA pathway (for artemisinic acid production) was used. *IDI* isopentenyl-diphosphate isomerase, *IspA/ERG20* FPP synthase, *ValCS* valencene synthase, *ZSS1* α-humulene synthase, *CDS* cadinene synthase, *ADS* amorpha-4,11-diene synthase, *CYB5* cytochrome b_5, *ADH* artemisinic alcohol dehydrogenase, *ALDH* artemisinic aldehyde dehydrogenase

oxidases [390, 391]. When coexpressed with valencene synthase and reductase 1 (ATR1), CYP71AV8, and CYP706M1 enabled the production of nootkatol and nootkatone at low levels in yeast (40 and 144 μg L^{-1}, respectively). An interesting observation made with CYP706M1 was that nootkatone was produced in vivo exclusively in the absence of a second *n*-dodecane phase.

A more complex example is the production of 8-hydroxy-α-humulene. This is the direct precursor of zerumbone, which is contained in *Zingiber zerumbet* ("shampoo ginger"). 8-Hydroxy-α-

humulene was shown to be a promising chemopreventive agent for suppressing atherosclerosis, HIV, as well as tumors [392, 393]. Its production from mevalonate could be achieved in *E. coli* via a combination of an engineered lower MVA pathway, α-humulene synthase, CYP71BA1, and different CPRs. Maximum product accumulation of 0.4 mg L^{-1} occurred when ginger CPR1 was coexpressed with CYP71BA1. 37.7 mg L^{-1} of remaining α-humulene indicate that the subsequent hydroxylation step represents a limitation in this pathway.

Improved sesquiterpenoid production of approximately 100 mg L^{-1} 8-hydroxycadinene, which is a precursor of the potential anticancer drug gossypol, was accomplished in *E. coli*. These metabolically engineered cells contained a heterologous MVA pathway and overexpressed FPP synthase along with cadinene synthase, CYP706B1 from *Gossypium arboretum* (Cotton), and a surrogate CPR from *Candida tropicalis* [81].

The most perfected example for the microbial production of a plant-based secondary metabolite is the "Artemisinin Success Story." Artemisinin is part of the artemisinin-based combination therapies (ACT) against malaria, which are recommended by the World Health Organization [394]. The natural source is the sweet wormwood plant *A. annua*, which is grown mostly in China and Vietnam. However, the availability and market supply of this drug is hampered by varying harvests and long production periods (~14 months). The production of the precursor artemisinic acid using engineered yeast was first described by Jay Keasling and coworkers in 2006 [80]. The endogenous MVA pathway and subsequent prenylation steps in *S. cerevisiae* were optimized to increase the production of FPP, which was converted to the sesquiterpene amorpha-4,11-diene by coexpression of amorphadiene synthase. This pathway was extended by the introduction of CYP71AV1 and its cognate CPR from *A. annua* for further amorpha-4,11-diene oxyfunctionalization yielding artemisinic acid via the intermediates artemisinic alcohol and artemisinic aldehyde. Using this engineered whole cell system, titers of up to 100 mg L^{-1} artemisinic acid could be produced. An analogously engineered *E. coli* strain produced up to 325 mg L^{-1} artemisinic acid. Addition of an *n*-dodecane phase resulted in the accumulation of the intermediates artemisinic alcohol and artemisinic aldehyde, while full oxidation of the sesquiterpene precursor was only observed in the absence of a second phase [81], similar to the case of nootkatone production discussed above. This effect was attributed to the ability of the organic overlay to extract both the product artemisinic acid and the intermediates artemisinic alcohol and artemisinic aldehyde.

Recently, further improved yeast systems were reported [395]. These included, among others, CYP71AV1 and an alcohol dehydrogenase and aldehyde dehydrogenase (Adh1 and AldH1) from *A. annua* for the respective conversion of artemisinic alcohol and aldehyde [396]. Titers of artemisinic acid of up to 25 g L^{-1} were achieved in fermentation experiments and could be further converted to artemisinin by means of classical chemistry or photochemistry [395]. This semisynthetic process is now used at Sanofi for the industrial production of artemisinin.

8.7.1.1.3 Diterpenoids

E. coli strains engineered to produce labdane-type diterpenes by a modular approach were the basis for the production of the corresponding diterpenoids [397]. For this, the *E. coli* MEP pathway was exploited in conjunction with coexpression of GGPP synthase from *Abies grandis* and a first diterpene cyclase to produce either *syn*- or *ent*-copalyl pyrophosphate (CPP). These compounds are further converted by a second terpene cyclase to the respective labdane-related diterpenes (Fig. 8.14). For instance, based on such an approach, the precursor of the antifungal phytocassanes A-E, namely 11-hydroxy-*ent*-cassadiene, was produced by coexpression of CYP76M7 in a strain capable of *ent*-cassadiene accumulation [398]. Multifunctional CYP99A3 coexpressed in *syn*-pimaradiene- and *syn*-stemodene-producing strains catalyzed oxidations at the C19 moiety to sequentially form the respective alcohols, aldehydes, and acids, which are precursors of the chemotherapeutic momilactone B [399]. Introduction CYP71Z6 into an *ent*-isokaurene-pro-

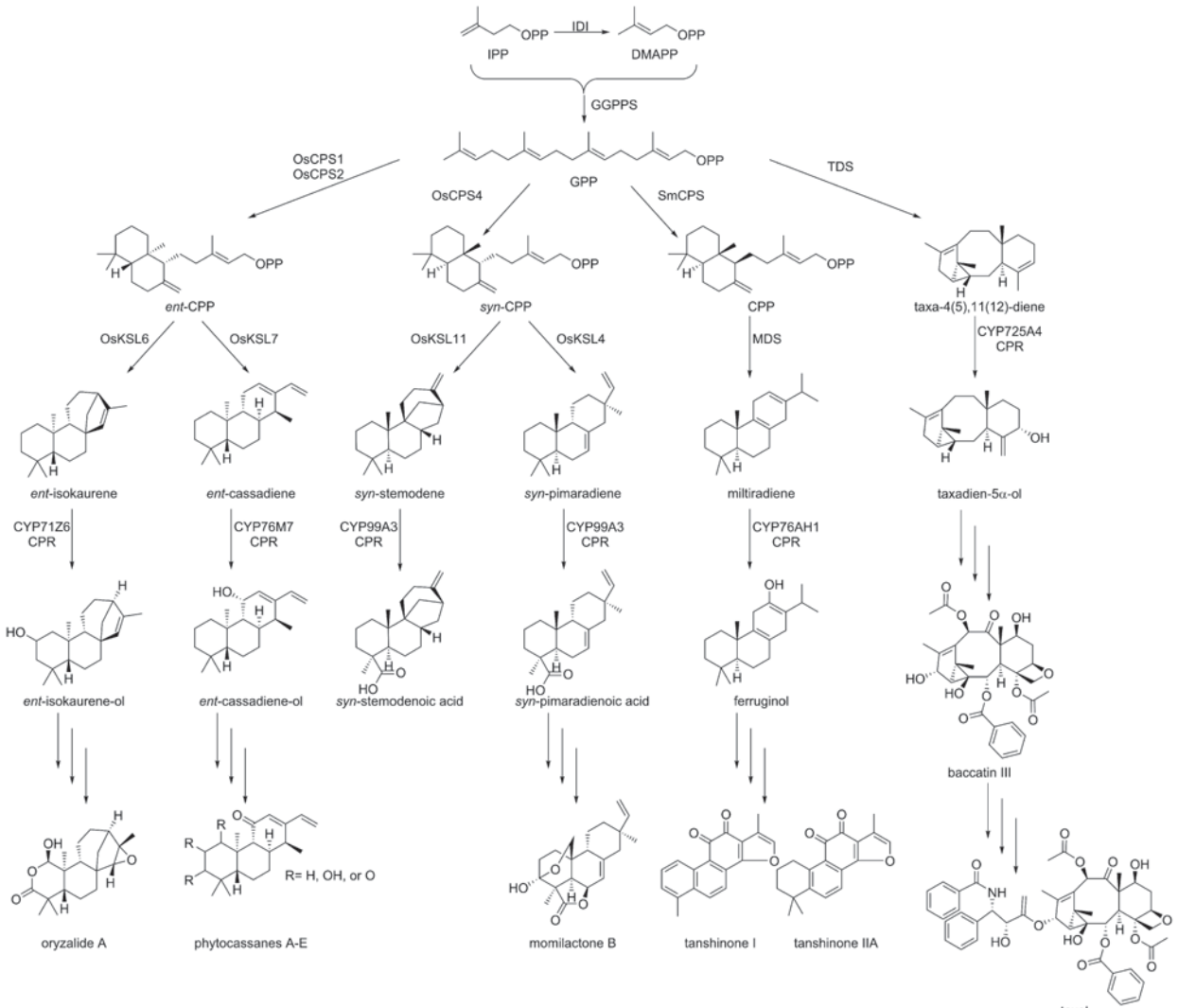

Fig. 8.14 Engineered pathways for the production of diterpenoids. Isopentenyl pyrophosphate (*IPP*) and dimethylallyl pyrophosphate (*DMAPP*) are derived either from the native or optimized methylerythritol phosphate (*MEP*) pathway in the case of diterpenoid phytoalexins and taxadien-5-α-ol, respectively. For ferruginol production in yeast, the precursors were provided by the internal mevalonate (*MVA*) pathway. *IDI* isopentenyl-diphosphate isomerase, *GGPPS* GGPP synthase, *OsCPS Oryza sativa* CPP synthase, *SmCPS Salvia miltiorrhiza* CPP synthase, *OsKSL Oryza sativa* kaurene synthase like diterpene cyclases, *MDS* miltiradiene synthase, *TDS* taxa-4(5),11(12)-diene synthase

ducing strain led to the production of 2-hydroxy-*ent*-isokaurene, which is a precursor of oryzalide A [400].

The recombinant production of ferruginol, which is the precursor of the anticancer compounds tanshinones, was achieved by using CYP76AH1 in a strain optimized for miltiradiene accumulation and reached titers of 10.5 mg L^{-1} [401]. In addition, the strain contained an artificial fusion protein of GGPP synthase and FPP synthase as well as a truncated HMGR and a fused synthetic mitiradiene–CPP synthase module (Fig. 8.14).

The implementation of an engineered pathway for paclitaxel (referred to as taxol) precursor production in *E. coli* including a P450 was reported by the group of Greg Stephanopoulos [402]. Taxol and its derivatives are mitotic inhibitors used as chemotherapeutic agents. Taxol was first isolated from the bark of the pacific yew tree, *Taxus brevifolia,* and later also found in other *Taxus* species. The demand for taxol is currently covered by semisynthetic routes starting with the intermediates 10-deacetylbaccatin III or baccatin III extracted from *Taxus* needles (Idena) or plant cell culture (Bristol-Myers-Squibb/Phyton Inc.). However, both strategies are labor and time intensive and the first might be subject to seasonal fluctuations with regard to product yield, so that microbial production might be a competitive alternative. In contrast to the successful application of the MVA pathway for monoterpenoid and sesquiterpenoid production, taxadiene-5-α-ol was produced by engineering the endogenous MEP pathway of *E. coli*. Furthermore, the universal isoprenoid precursors IPP and DMAPP were converted to geranylgeranyl pyrophosphate (GGPP) by coexpression of GGPP synthase. GGPP in turn serves as substrate for coexpressed taxadiene synthase forming the unfunctionalized taxol precursor taxa-4(5),11(12)-diene at titers of approximately 1 g L^{-1}. The researchers further demonstrated the implementation of taxadiene-5-α-hydroxylase (CYP725A4) catalyzing the first oxidation step in taxol biosynthesis (Fig. 8.14), yielding taxadien-5-α-ol at titers of 58 mg L^{-1}. Interestingly, taxadiene-5-α-hydroxylase was employed as an artificial fusion protein consisting of CYP725A4 and *Taxus* CPR. Although the results of this study represent a milestone in taxol production, the way towards taxol-producing microorganisms remains long; it presumably requires 17 more enzymatic steps, of which several are catalyzed by P450s, and some of the involved enzymes are still unknown [374, 403].

8.7.1.1.4 Triterpenoids

Triterpenoids have lately attracted great attention. The biosynthetic pathways for these compounds are encoded by clustered genes in plants. Besides their natural role as secondary metabolites for the optimization of plant–environment interactions, triterpenoids are also considered as pharmaceuticals and pesticides [376, 404]. Consequently, an increasing number of attempts to produce triterpenoids in *S. cerevisiae* have been reported (Fig. 8.15). On the basis of strains producing the triterpene core structures β-amyrin, α-amyrin, and lupeol, it was shown that *Medicago truncatula* CYP716A12, *Vitis vinifera* CYP716A15, as well as CYP716A17 were all multifunctional enzymes. Together with the *Lotus japonicus* CPR, each of the enzymes was capable of catalyzing the three-step oxidations of β-amyrin to oleanolic acid, α-amyrin to ursolic acid, and lupeol to betulinic acid in yeast [405]. Further, transgenic yeast strains producing soyasapogenol B and gypsogenic acid were constructed by combinatorial biosynthesis, employing β-amyrin synthase, CPR, as well as CYP93E2 and CYP72A61v2 or CYP716A12 and CYP72A68v2 from *M. truncatula* [406].

In a different study, the roles of CYP708A2, CYP705A1, and CYP71A16 from *A. thaliana* in triterpenoid metabolism were unraveled by introducing these enzymes in yeast strains producing thalianol, arabidiol, and marneral. Using the internal 2,3-oxidosqualene pool, these metabolically engineered yeast strains produced the corresponding oxygenated derivatives in the range of mg L^{-1} [407]. A recent study combines an increased carbon flux through the native MVA pathway resulting from the expression of a truncated HMG-CoA reductase, with the introduction of yeast squalene synthase and plant 2,3-oxidosqualene cyclases to produce a β-amyrin and dam-

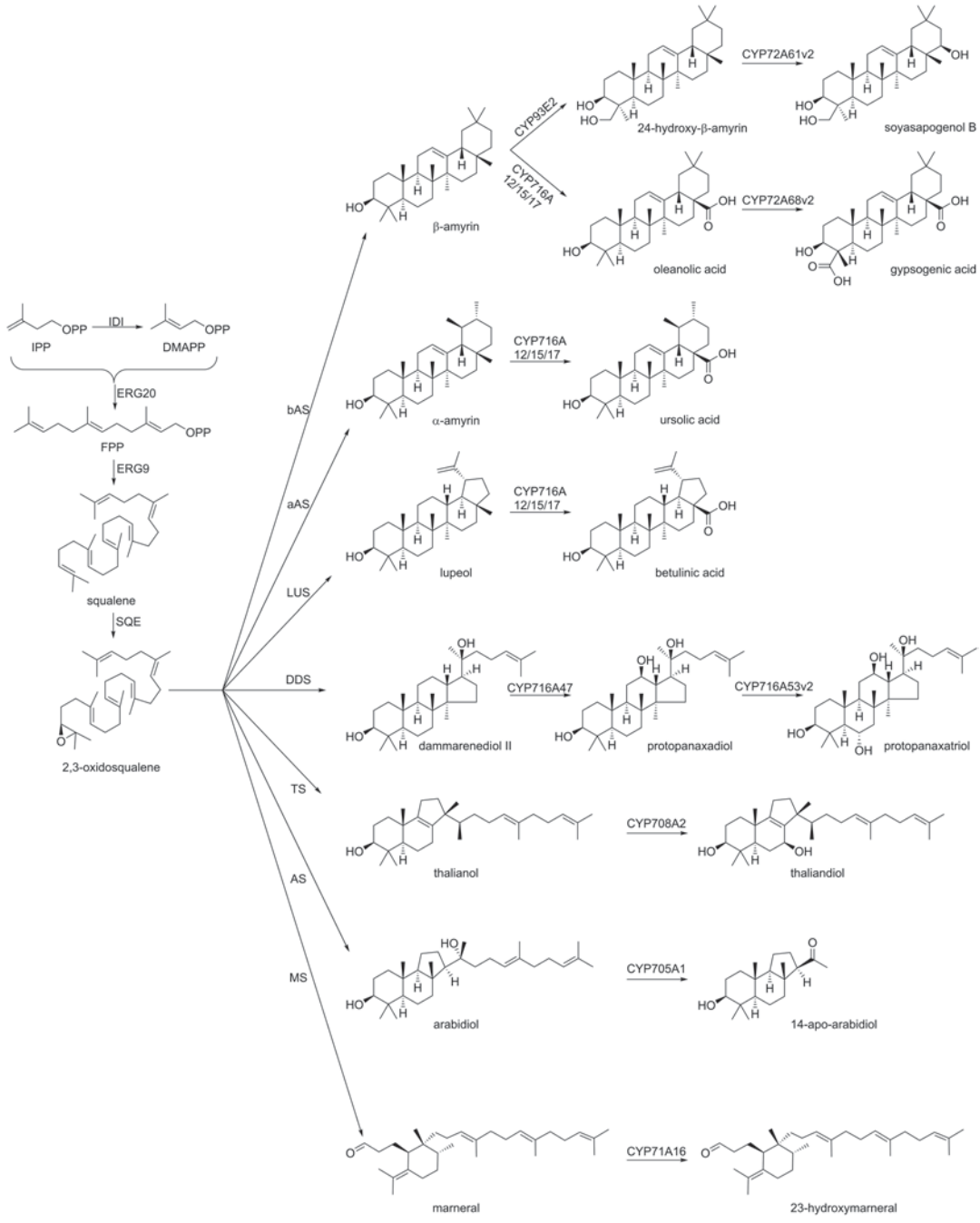

Fig. 8.15 Engineered pathways for the production of triterpenoids in *S. cerevisiae*. Isopentenyl pyrophosphate (*IPP*) and dimethylallyl pyrophosphate (*DMAPP*) were provided either by an optimized or native mevalonate (MVA) pathway. Although not indicated in this figure, in all cases, a cytochrome P450 diflavin reductase (CPR) of *A. thaliana* served as redox partner for the indicated P450s. *ERG20* FPP synthase, *ERG9* squalene synthase, *SQE* squalene epoxidase, *bAS* β-amyrin synthase, *aAS* α-amyrin synthase, *LUS* lupeol synthase, *DDS* dammarenediol II, *AS* arabidiol synthase, *MS* marneral synthase

marenediol II [408]. Furthermore, simultaneous employment of CYP716A12, CYP716A47, and CYP716A53v2 and a CPR led to the production of 21.4 mg L^{-1} oleanolic acid, 17.2 mg L^{-1} protopanaxadiol, and 15.9 mg L^{-1} protopanaxatriol, respectively. This strain might serve in the future as basis for the production of a broad range of ginsenosides by coexpression of glycosyltransferases.

8.7.1.1.5 Carotenoids

Carotenoids are derived from tetraterpenes and are assumed to provide health benefits by decreasing the risk of disease, particularly in cancers and eye disease. For the production of hydroxylated carotenoids, along with functional identification of the involved enzymes, P450s from *Oryza sativa* were introduced in carotenoid-producing *E. coli* strains (Fig. 8.16) [409]. CYP97A4 catalyzed the conversion of β-carotene to β-cryptoxanthin and zeaxanthin. In contrast, CYP97C2 converted only the ε-ring substrates δ-carotene and ε-carotene.

8.7.1.2 De Novo Synthesis of Flavonoids

Flavonoids are a diverse family of plant polyphenols and of special interest due to their potential in the treatment of various human diseases. The first attempts to produce flavonoid precursors were accomplished by cloning of the flavanone pathway consisting of cinnamate-4-hydroxylase (CYP73A5) from *A. thaliana* together with 4-coumaroyl:CoA ligase (4CL), chalcone synthase (CHS), and chalcone isomerase (CHI) in *S. cerevisiae* [410]. The generated strain was able to convert cinnamic acid to 200 μg L^{-1} naringenin (Fig. 8.17). Naringenin could be further functionalized to produce apigenin (57 μg L^{-1}), when flavone synthase II (CYP93B) from *Antirrhinum majus* (snapdragon) and endogenous CPR were coexpressed in this yeast strain [411]. The same research group also used *E. coli* to produce hydroxylated flavonoids by introducing 4CL, CHS, CHI, flavanone 3β-hydroxylase (FHT), flavonol synthase (FLS), and flavonoid 3′,5′-hydroxylase (CYP75A) fused to its CPR redox partner (Fig. 8.17) [412]. The production of highly functionalized flavonoids was strongly dependent on the choice of the growth medium and whether phenylpropanoid acids or flavanones were fed to the cells. Interestingly, reactions catalyzed by omitting the P450-catalyzed step, by using eriodictyol instead of naringenin as starting point of the reaction, proved to be more productive in flavonol synthesis.

8.7.1.3 De Novo Synthesis of Glucosinolates

Glucosinolates are amino acid derived and sulfur-rich secondary metabolites, which are characteristic for cruciferous plants. Glucosinolates have been linked to a number of benefits to human health, such as prevention of cardiovascular diseases and reduction of the risk of developing cancer [413]. In a proof-of-concept study, tryptophan-derived indolylglucosinolates (IGs) were produced in recombinant yeast by stepwise integration of catalytic enzymes to yield a seven-step pathway for the production of indolmethyl-glucosinolate (Fig. 8.18) [414]. The first two steps of this pathway included CYP79B2 and CYP83B1 from *A. thaliana*, which catalyze the conversion of tryptophan to indolylacetaldoxime and further to indolylacetonitrile oxide. Coexpression of ATR1, glutathione S-transferase (GSTF9), γ-glutamyl peptidase (GGP1), C-S lyase (SUR1), a glycosyltransferase (UGT74B1), and a sulfotranferase (ST5a; all from *A. thaliana*) showed that production of IG is possible, even though titers were low (1.07 mg L^{-1}).

8.7.1.4 De Novo Synthesis of Alkaloids

Work on the microbial production of plant alkaloids has been mainly focused on benzylisoquinoline alkaloids (BIAs), which are a diverse class of metabolites with a broad range of pharmaceutical activities. Hawkins and Smolke engineered yeast to express combinations of enzymes from plants and humans for the production of a wide array of BIAs (Fig. 8.19) [415]. As early steps of BIA biosynthesis were not identified at that time, the commercially available but unnatural substrate (*R,S*)-norlaudanosoline was converted to the key intermediate (*R,S*)-reticuline by three consecutive methyl transfer steps. (*S*)-reticuline

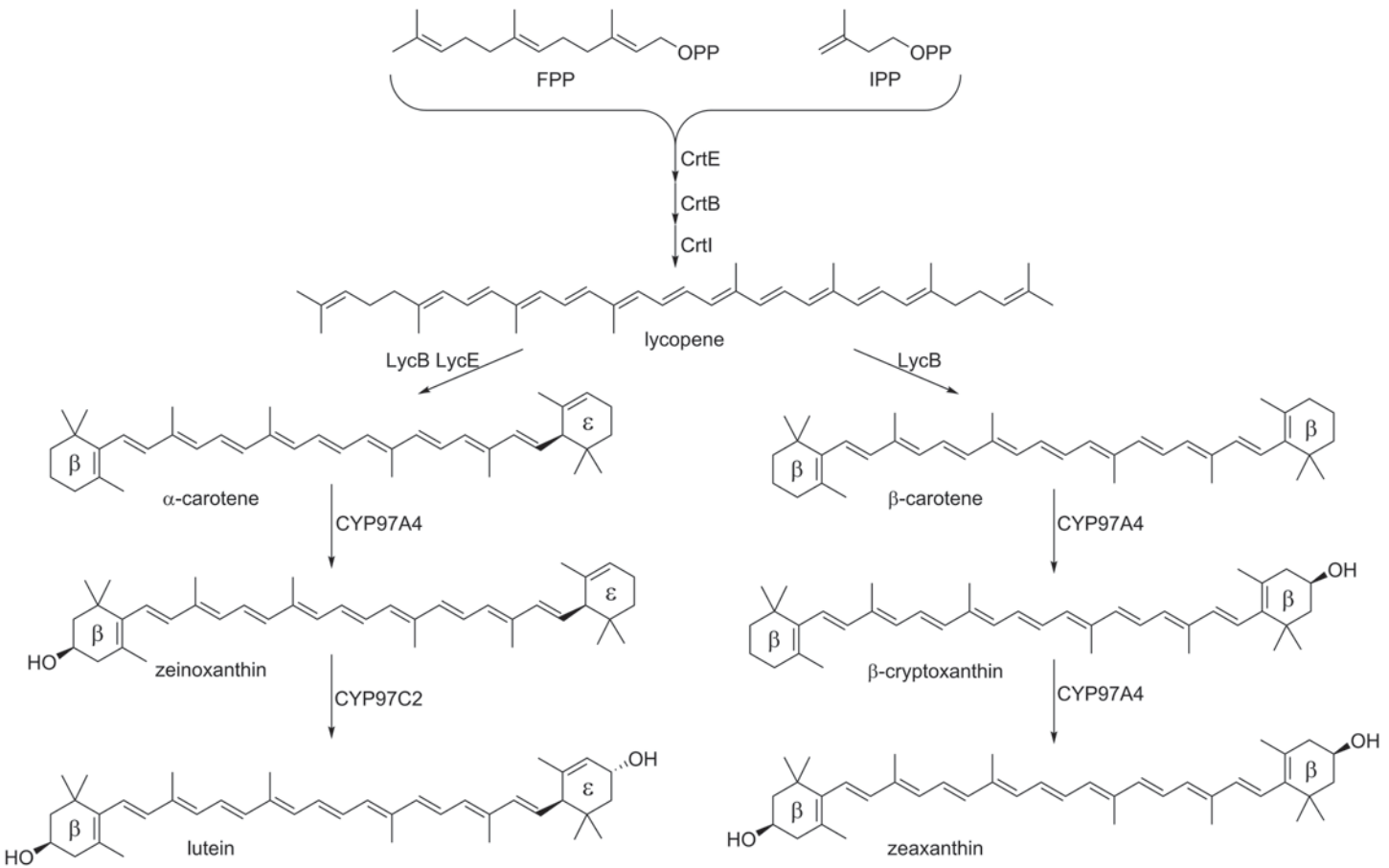

Fig. 8.16 Pathways for the production of hydroxylated carotenoids in *E. coli*. Isopentenyl pyrophosphate (*IPP*) and farnesyl diphosphate (*FPP*) are produced via internal methylerythritol phosphate (MEP) pathway and FPP synthase. *CrtE* GGPP synthase, *CrtB* phytoene synthase, *CrtI* phytoene dehydrogenase, *LycB* lycopene β-cyclase, *LycE* lycopene ε-cyclase

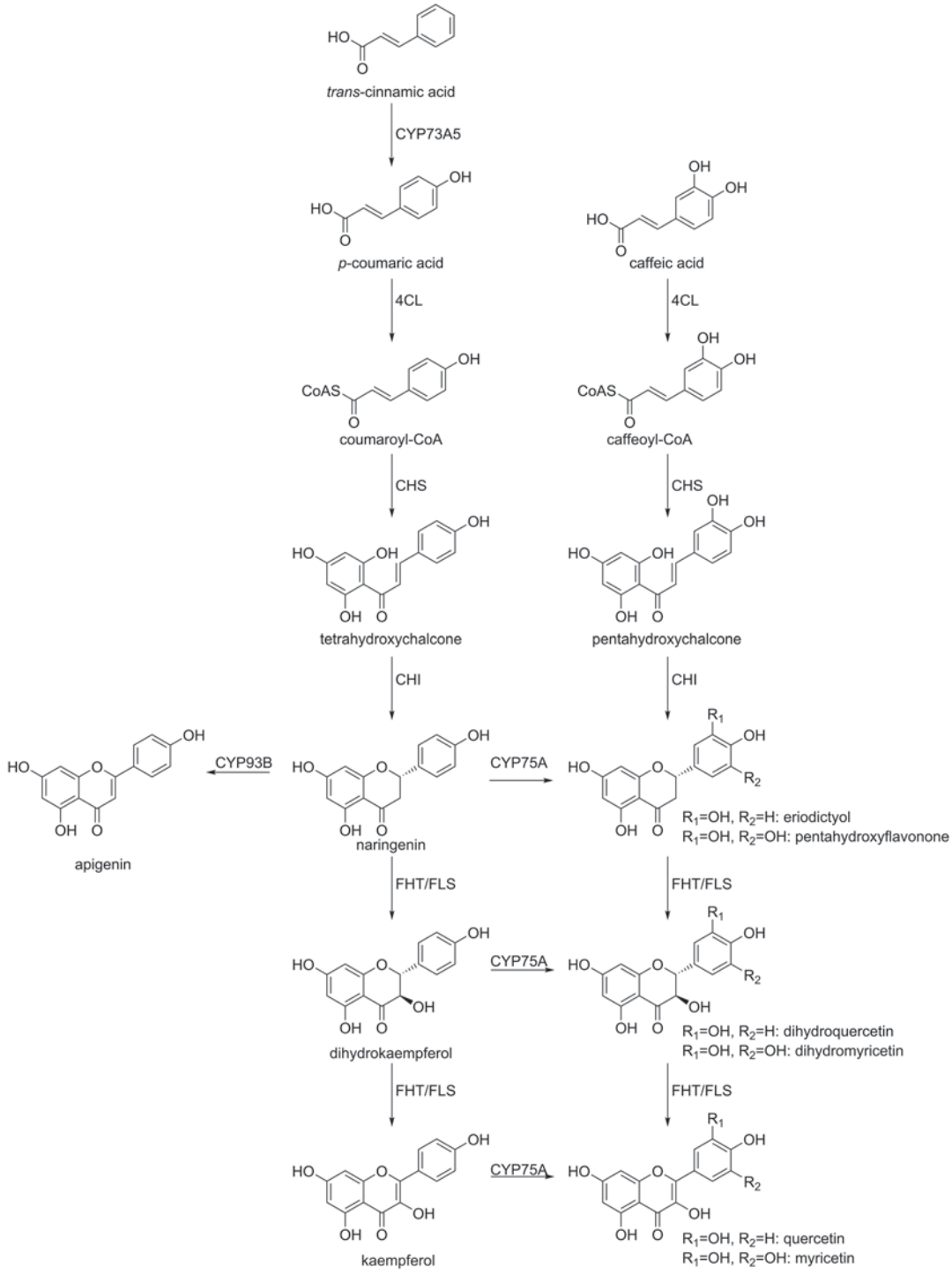

Fig. 8.17 Schematic representation of pathways for the production of flavonoids. *4CL* 4-coumaroyl:CoA ligase, *CHS* chalcone synthase, *CHI* chalcone isomerase, *FHT* flavanone 3β-hydroxylase, *FLS* flavonol synthase

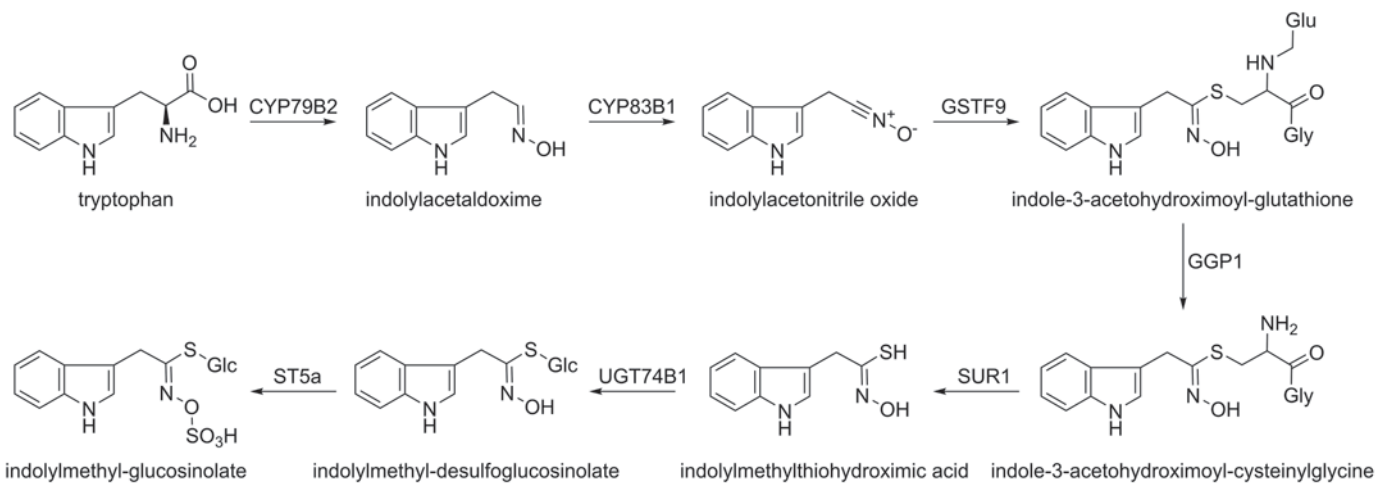

Fig. 8.18 Seven-step pathway introduced in yeast for the accumulation of indolmethyl-glucosinolate. The precursor tryptophan is produced by the native yeast pathway

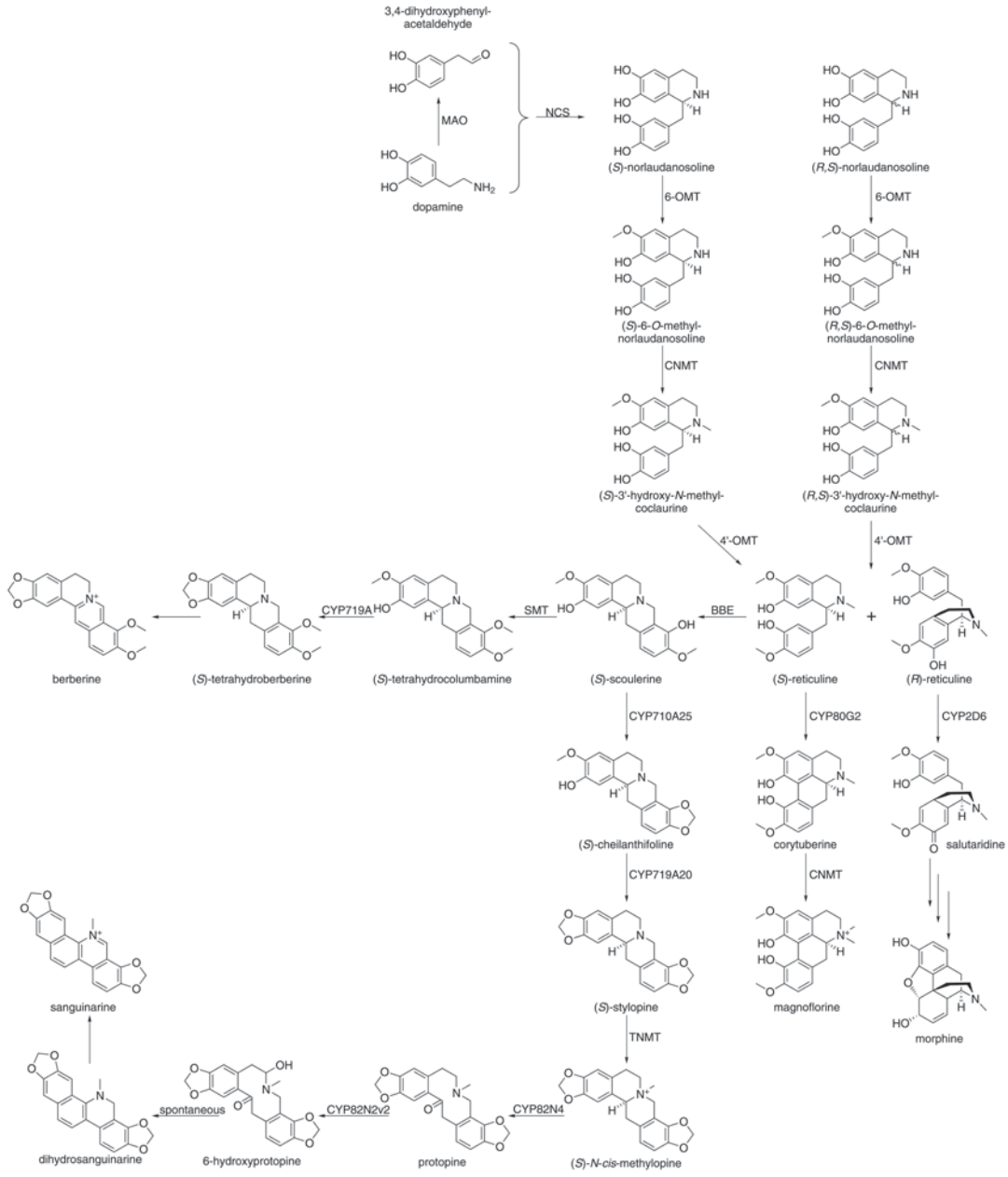

Fig. 8.19 Natural and nonnatural pathways for the production of the BIA branch point metabolite reticuline as well as further downstream metabolites. The final products berberine, morphine, and sanguinarine are also indicated. Unlabeled arrows indicate that the corresponding enzymes have not been identified so far. *MAO* monoamine oxidase, *NCS* norcoclaurine synthase, *6-OMT* 6-*O*-methyltransferase, *CNMT* coclaurine-*N*-methyltransferase, *4′-OMT* 3′-hydroxy-N-methylcoclaurine-4′-O-methyltransferase, *BBE* berberine bridge enzyme, *SMT* (*S*)-scoulerine 9-O-methyltransferase, *TNMT* tetrahydroprotoberberine *cis*-*N*-methyltransferase

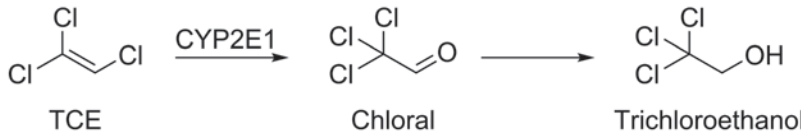

Fig. 8.20 CYP2E1-mediated transformation of trichloroethylene (*TCE*)

could be further converted by the joined action of berberine bridge enzyme (BBE), (*S*)-scoulerine 9-*O*-methyltransferase (SMT), and canadine synthase (CYP719A1) to yield the berberine precursor (*S*)-tetrahydroberberine [415]. Interestingly, a lower gene dose of the surrogate reductase ATR1 as a result of chromosomal integration turned out to be more favorable for (*S*)-tetrahydroberberine production. An estimated titer of 30 mg L^{-1} from a substrate concentration of 4 mM (approx. 1150 mg L^{-1}) was achieved, while conversion was in the range of 1–2%. The accumulation of several reaction intermediates indicated that flux limitations were still present. An alternative route converting the formed (*R*)-reticuline to yield the morphine precursor salutaridine required the use of human CYP2D6 because the native plant enzyme catalyzing this step has not been identified so far. However, the relatively low activity of CYP2D6 on (*R*)-reticuline led to titers of approximately 20 mg L^{-1} from 4 mM substrate.

In a recent study, a ten-gene pathway for the synthesis of the BIA dihydrosanguinarine was reconstituted in *S. cerevisiae* (Fig. 8.19) [416]. (*S*)-reticuline was produced in the same way as in the aforementioned study and then further converted to dihydrosanguinarine by five additional steps, with a final product yield of 1.5% from 10 μM substrate. Importantly, four of these reaction steps were catalyzed by P450s, which included cheilanthifoline synthase (CFS; CYP710A25), stylopine synthase (SPS; CYP719A20), (*S*)-*cis*-N-methylstylopine 14-hydroxylase (MSH; CYP82N4), and protopine 6-hydroxylase (P6H, CYP82N2v2).

Another study features the use of *E. coli* and *S. cerevisiae* for BIA production [417]. While *E. coli* was employed for the production of (*S*)-reticuline from 5 mM dopamine, *S. cerevisiae* cells expressing CYP80G2 and coclaurine-*N*-methyltransferase (CNMT) (both enzymes difficult to express in *E. coli*) were added at a later stage to synthesize 7.2 mg L^{-1} magnoflorine with a yield of 2.2%.

8.7.2 Transgenic Plants

8.7.2.1 Phytoremediation

Phytoremediation is the use of plants to clean up environmental pollution. To overcome limitations like the slow rate of removal or incomplete metabolism, new enzymatic activities are introduced in plants by genetic engineering. In several cases, P450s of bacterial or mammalian origin were expressed in plants in order to remediate polluted soil, groundwater, or air [418].

Expression of the human CYP2E1 in hydroponically grown tobacco enhanced the metabolism of the volatile hydrocarbon trichloroethylene (TCE) up to 640-fold. The oxidation product 2,2,2-trichloroacetaldehyde (chloral) which was generated by the P450 was further metabolized in the plant to the corresponding alcohol (Fig. 8.20) [419]. In a later study, transgenic poplar rather than tobacco was used due to its faster growth, larger size, and more extensive root system. The best performing transgenic lines expressing rabbit CYP2E1 showed more than 100-fold higher TCE-metabolism rates than the control. Due to the broad substrate spectrum of CYP2E1, improved removal rates could also be observed for other environmental pollutants, such as chloroform, carbon tetrachloride, and vinyl chloride. Interestingly, volatile TCE could also be removed from polluted air by whole transgenic plants [420].

In addition, phytoremediation of herbicides can be enhanced by transgenic plants expressing P450s. Rice plants expressing human CYP1A1, either separately or in conjunction with CYP2B6 and CYP2C19, showed a high resistance to a broad range of herbicides with different modes of action, including atracine, metolachlor, norflurazon and mixtures thereof [421–423].

Phytoremediation has also been achieved for the military explosive hexahydro-1,3,

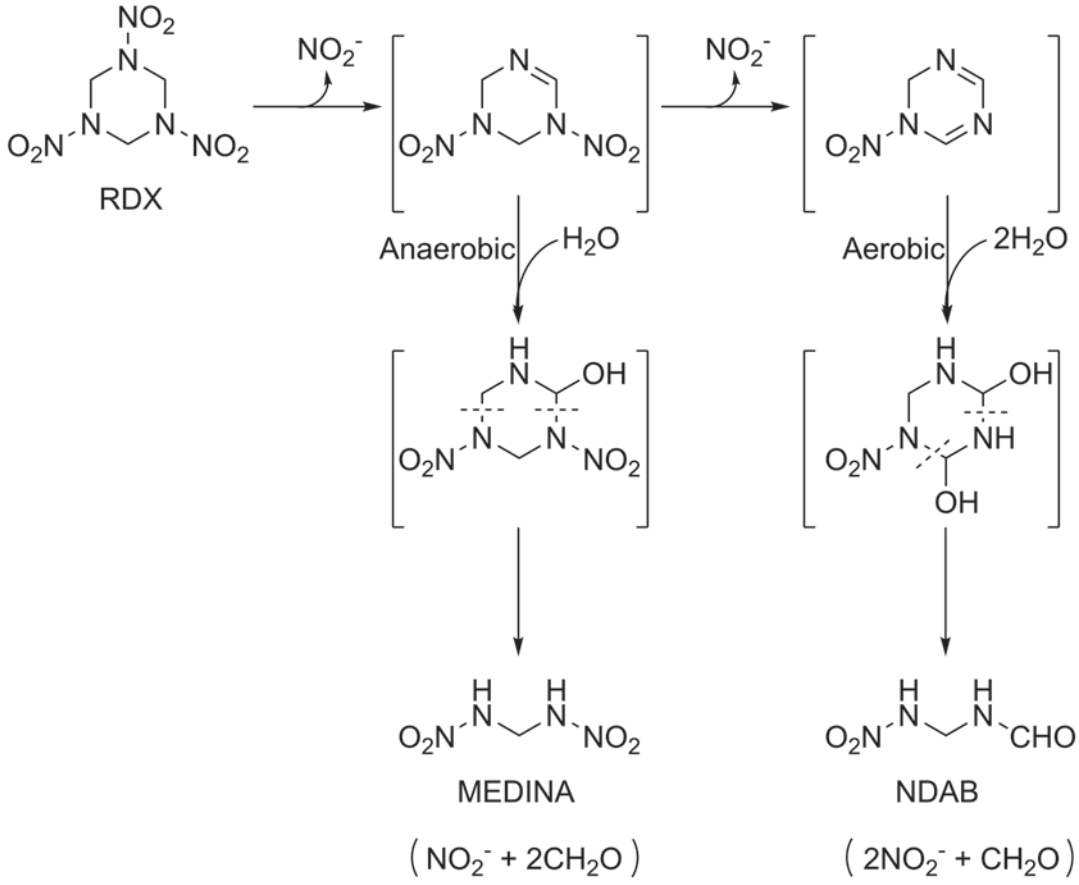

Fig. 8.21 Proposed mechanism for the CYP177A1-mediated degradation of royal demolition explosive (RDX) under anaerobic and aerobic conditions

5-trinitro-1,3,5-triazine (royal demolition explosive, RDX). This compound is toxic not only to mammalians but also to plants. Consequently, it cannot be degraded by classical phytoremediation. Nevertheless, the use of CYP177A1 (XplA) which was originally found in *Rhodococcus* sp. isolated from RDX-contaminated sites allowed the degradation of RDX. CYP177A1 displays an unusual structure with an N-terminal Fld domain fused to a C-terminal P450 domain (see Fig. 4.9, Sect. 4.1). Although the complete mechanism has not been elucidated so far, it has been shown that CYP177A1 catalyzes the single or double denitration of RDX under anaerobic and aerobic conditions, respectively. Hydration probably leads to unstable intermediates, which decompose to nitrite and formaldehyde and either methylendinitramine (MEDINA) or 4-nitro-2,4-diaza-butanal (NDAB; Fig. 8.21). However, the reaction occurs more efficiently at hypoxic conditions. Axenic liquid cultures of *A. thaliana* expressing CYP177A1 detoxified media containing 180 µM RDX within 5 days. When grown on contaminated soil, the same plants exhibited no signs of RDX-toxicity or growth deficiency, whereas wild-type plants did [171]. Engineered plants co-expressing the P450 along with its native reductase XplB operated 30 times faster in terms of RDX removal [424].

8.7.2.2 Reduction of Toxic Secondary Metabolites

Besides the expression of P450s in plants, as shown in the case of phytoremediation, also gene silencing plays an important role, e.g., to prevent the production of endogenous carcino-

genic or antinutritional secondary metabolites. A successful example is the suppression of nicotine conversion to nornicotine, a direct precursor in the synthesis of the potent carcinogen N′-nitrosonornicotine. Knockout of the nicotine N-demethylases (CYP82E4, CYP82E5v2 and CYP82E10) in tobacco resulted in significantly reduced nornicotine levels compared to those found in conventional tobacco cultivars [425, 426]. Recently, the biosynthesis of antinutritional steroidal glycoalkaloids (SGAs), such as α-solanine, α-chaconine, or α-tomatine in solanaceous crops was elucidated. SGAs cause gastrointestinal and neurological disorders and, at high concentrations, may be lethal to humans. By silencing the *GAME4* gene encoding CYP88 the accumulation of SGAs was prevented in potato tubers and tomato fruit [427].

8.7.2.3 Ornamental Plants

An actual industrial application promoted by Suntory Ltd. (Japan) and Florigene Pty Ltd. (Australia) is the exploitation of P450s involved in biosynthesis of delphinidin-type anthocyanins for the production of roses and carnations with nonnatural colors that cannot be achieved by classical breeding [428]. Expression of the flavonoid 3′,5′-hydroxylase (F3′,5′-H; CYP75A) and dihydroflavonol reductase (DFR) from *Petunia* in *DFR*-deficient variants led to an exclusive accumulation of delphinidin derivatives and a significant color shift towards blue (Fig. 8.22) [429].

The resulting flower FLORIGENE Moondust was the first commercially available floricultural crop in the world. Introduction of pansy F3′,5′-H instead of its *Petunia* homolog, either alone or in combination with CYP75A of *Salvia* sp., increased delphinidin levels, yielding dark violet carnations (FLORIGENE Moonshadow and FLORIGENE Moonique) [430, 431]. Up to now, carnations with several shades have been developed from suitable varieties through the expression of different genes in diverse genetic arrangements and the customized downregulation of DFR and flavonoid 3′-hydroxylase (F3′-H; CYP75B) genes [432].

For the generation of roses with higher delphinidin content cultivars with higher petal vacuolar pH and flavonol amounts as well as lower F3′-H activity were selected and transformed with F3′,5′-H from pansy [433]. Suntory blue rose *Applause* has been commercialized in Japan since 2009 (Fig. 8.23). To achieve a more pansy-like blue color, further modifications regarding the production of strong copigments and elevation of vacuolar pH are still needed.

8.8 Conclusions and Perspectives

Cytochrome P450 enzymes catalyze a vast variety of chemical transformations and accept a broad spectrum of substrates. Their ability to perform highly selective oxidation reactions at unactivated C–H bonds at room temperature and under normal pressure demonstrates the sustainability of P450 biocatalysts. Therefore, P450s are considered as attractive candidates for the synthesis of valuable compounds. However, as generally recognized, the use of P450s in industrial processes is still limited because of their complexity, low activity, and the need for the reducing cofactors NAD(P)H and redox partner proteins, which generally result in low product yields. Over the last two decades, our fundamental understanding of P450 systems has greatly improved and tremendous progress has been made in making these systems more suitable for industrial application. Bioprocesses for industrial production of fine chemicals are considered to require space–time yields of at least 0.1 g L^{-1} h^{-1} [434]. To date, most of the reported P450-based biocatalytic systems do not fulfill this requirement. However, for the production of pharmaceutical compounds, acceptable process productivities may be as low as 0.001 g L^{-1} h^{-1} [435]. This value is already met by several reported P450 biocatalysts.

The aspect of economic feasibility of biotechnological processes involving P450s has been studied by Andreas Schmid and colleagues [435]. An operational window for twelve reported P450-based processes was analyzed and compared to the industrially relevant space–time yields. Interesting in this context is the artificial multienzyme cascade process involving CYP71AV1 from *A.*

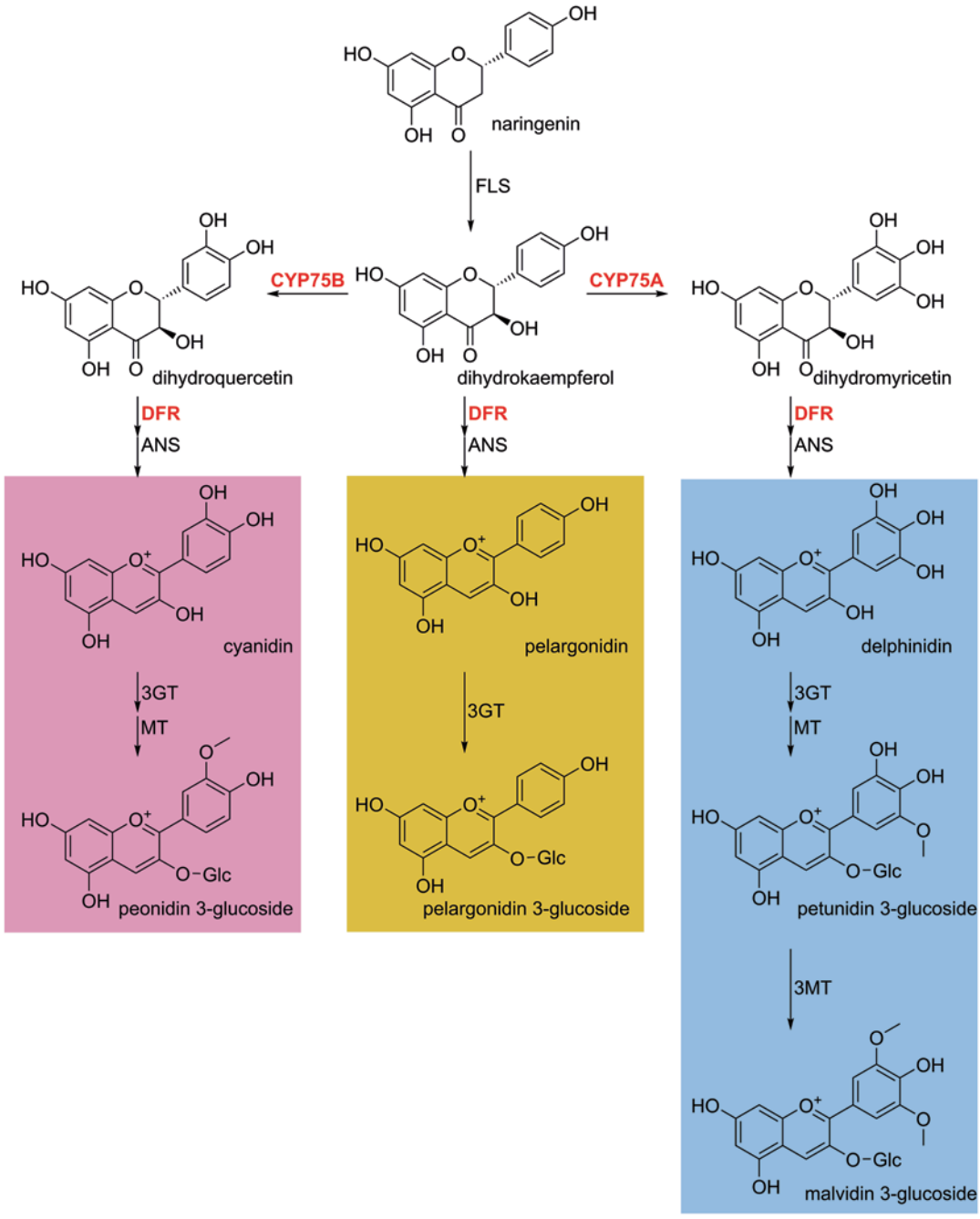

Fig. 8.22 Flavonoid biosynthetic pathways relevant for flower colors. Typical colors resulting from each of the anthocyanins are indicated by the *colored boxes*. Other factors affecting the color like copigments are not represented. Modified activities are highlighted in *red*. *DFR* dihydroflavonol 4-reductase, *ANS* anthocyanidin synthase, *3GT* anthocyanidin 3-*O*-glucosyltransferase, *MT* methyltransferase

Fig. 8.23 The blue rose *Applause* developed by Suntory. (Reproduced with permission of Suntory Flowers Limited, Tokyo, Japan)

annua for the production of artemisinic acid in engineered *S. cerevisiae, reported in 2006* [80]. This production system hardly fulfilled the minimal requirements defined for pharmaceutical compounds at that time [435]. However, further improvements of this system led in 2013 to artemisinic acid concentrations of up to 25 g L^{-1} in fermentation experiments [395, 436]. A process based on the engineered *S. cerevisiae* strain producing artemisinic acid is now used for the industrial production of artemisinin at Sanofi (http://www.rsc.org/chemistryworld/2013/04/sanofi-launches-malaria-drug-production). This example perfectly demonstrates that recombinant protein technology combined with the methods of synthetic biology, metabolic engineering, and downstream processing opens up completely new perspectives for P450-based processes. The ability of P450s to catalyze highly selective reactions on complex molecules, which can be combined with additional chemical reactions to create chemo-enzymatic processes for the production of molecules of interest (as in the case of artemisinin), makes P450s interesting tools for the synthesis and modification of natural compounds based on renewable feedstocks.

Artemisinin is a component of the artemisinin-based combination malaria therapies [394]. In 2010, more than 200 million cases of malaria, and at least 655,000 malaria-related deaths were reported [437]. Obviously, a constant and cheap source of artemisinin is required to support a cost-effective treatment [395]. In addition, new effective agents against cancer as well as new antibiotics and new anti-inflammatory compounds are required. For instance, the annual production of steroid drugs has exceeded 1,000,000 t and the global market is around US$ 10 billion [318]. The demand for drug metabolites is also rising, while at the same time safety regulations are tightened. In 2008, the US Food and Drug Administration (FDA) issued a "Guidance for Industry: Safety Testing of Drug Metabolites," which defines that drug metabolites present in circulation at a fraction >10% (formerly >25%) of the parent drug must undergo safety testing [438, 439]. A similar "Guideline on the Investigation of Drug Interactions" was issued in June 2012 by the Committee for Human Medicinal Products (CHMP) of the European Medicines Agency (EMA) [440].

The demand for high-value oxyfunctionalized fine chemicals has also been increasing over the past years. In 2011, the estimated sales volume of the top ten flavor and fragrance industry leaders was US$ 22 billion (in comparison: US$ 16 billion in 2005) [441]. From an academic as well as commercial point of view, the increasing demands of high-value compounds represent strong market incentives for further developments in the field of biotransformation and de novo biosynthesis of oxygenated compounds. In cases where chemical synthesis or extraction from plants is not feasible, microbial systems could be a suitable alternative source for the production of such compounds. To achieve this goal, the use of P450 seems inevitable. A variety of secondary metabolites from different substance classes have already been produced by exploiting the synthetic

power of P450s. In addition to improved process engineering and control of the carbon flux by metabolic engineering, investigations of P450s and their dedicated redox partners have proven instrumental to metabolite production.

An important step towards improving the catalytic activities of P450 enzymes involves the covalent fusion of redox partner protein(s) to these enzymes. Many different approaches have been followed to create a multitude of artificial P450–redox partner fusion enzymes that catalyze a large variety of reactions. At first sight, it appears that no general rules for designing the optimal artificial P450 fusion construct can be deduced. On the other hand, it is evident that the proximity of the P450 domain to its redox partner(s) as well as a certain structural flexibility are important factors that contribute to efficient electron transfer.

Apart from activity improvement, enzyme engineering also allows the production of completely novel compounds that normally are not produced via natural routes [442]. To accelerate this process, the development and improvement of efficient microbial expression systems as well as high throughput screening methods are still crucial. The combination of such novel strategies enables exciting perspectives for future biocatalytic research and will certainly provide us with a completely new range of chemical compounds.

In this context, the modularity of P450 systems can be readily exploited. Strategies based on the easy exchange of modules (e.g., redox partners, NAD(P)H regeneration), which already have been established for a number of P450s, could easily be applied to other P450 systems as well, thereby significantly accelerating the setup of biotechnological processes.

Taken together, the elaborate strategies to improve the activity of P450 enzymes and to overcome their limitations make them excellent examples of biotechnological engineering. The extraordinary versatility of P450 enzymes that are used already today, as well as novel P450 enzymes or activities that will be discovered in the future, will undoubtedly be further developed and exploited for biotechnological application.

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Part II
Volume 2

Human Cytochrome P450 Enzymes

9

F. Peter Guengerich

9.1 History

The history of cytochrome P450 (P450) really began with studies on the metabolism of drugs, carcinogens, and steroids. The early research in these fields necessarily involved animal models, but the intent was always to understand the human systems in the context of the enzymes catalyzing the observed transformations.

A number of in vivo experiments in the realm of clinical pharmacology showed that drug metabolism was inducible [1–3] and varied among individuals [4]. Such phenomena were attributed to P450 enzymes after the development of research with experimental animals, but the molecular basis was unknown.

Early in vitro studies with human tissues were done but were difficult because of the limited availability of samples. It was possible to document the variability of human drug metabolism [5], although there were caveats about the quality of samples, etc.

The next phase of research was the purification of human P450s from liver microsomes. Some early efforts in this area were in the laboratories of Coon [6], Beaune [7], Kamataki [8], and Guengerich [9]. Highly purified P450s could be obtained, but analysis of catalytic specificity was generally limited to sets of a few typical substrates used with rat and rabbit P450 enzymes. However, some studies with warfarin oxidation were to provide insight, in that distinct activities were noted [10]. Clearly, multiple P450 existed in humans, as already appreciated in rats and rabbits. However, there was no clear indication how many human P450s might exist or how many would be involved in xenobiotic metabolism.

The human studies of Smith and his associates [11], along with others [12, 13], were very useful in that they first showed that the metabolism of an individual drug was genetically controlled. Monogenic control of the oxidation of a drug suggested that a single P450 would be dominant in its metabolism. This information led to a different plan to study human P450s: Purification was monitored with analysis of individual drug oxidation activities, rather than simply purifying the colored hemoproteins and then trying to establish their activities. The approach was, however, technically challenging in that individual fractions recovered from chromatography needed to be depleted of detergent, reconstituted with nicotinamide adenine dinucleotide phosphate-cyrochrome P450 (NADPH-P450) reductase, and monitored for activity using gas or liquid chromatography (LC). Nevertheless, with debrisoquine 4-hydroxylation and phenacetin *O*-deethylation, the approach yielded what are today termed P450s 2D6 and 1A2 [14]. Further work in this laboratory led to the purification of what are known today as P450s 2C8, 2C9 [15], 3A4 [16],

F. P. Guengerich (✉)
Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, 638 Robinson Research Building, 2200 Pierce Avenue, Nashville, TN, 37232-0146, USA
e-mail: f.guengerich@vanderbilt.edu

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2A6 [17], and 1A1 [18]. Work in other laboratories also yielded these same P450s purified from human liver [19–21] and P450s 2C19 [22] and 2E1 [23], plus P450 3A7 from fetal liver [24].

The purified P450s and their antibodies could be utilized to define the roles of individual P450s in the metabolism of individual drugs, carcinogens, and steroids. Other approaches developed during the 1980s included correlation of individual catalytic activities (in liver microsomes prepared from different individuals [25], or immunochemically determined levels of P450s [26, 27]) and the development/application of selective chemical inhibitors [14, 28–31].

Despite all of this progress in enzymology, there were still issues that could not be addressed easily. Some P450s were not expressed at levels high enough to be purified this way (and affinity chromatography methods were not effective). The need for large amounts of P450s in the future was a limitation. The development of recombinant DNA technology in the 1980s was yielding complementary DNAs (cDNAs) for P450s, but the only way to associate these with isolated P450 proteins was by N-terminal amino acid sequence analysis, using Edman degradation.

cDNA clones for many of the human P450s were rapidly isolated and used to determine nucleotide (and predictably amino acid) sequences, following the elegant work of Fujii-Kuriyama and his associates with rat P450 2B1 [32]. Much of the cDNA work was done by Gonzalez and his laboratory [33]. The cDNA work led to insight into the basis of the debrisoquine polymorphism described by Smith [11, 34].

After the success of cDNA cloning, practical heterologous expression of P450 enzymes was achieved in cells being CV-1 in origin and carrying the SV40 genetic material (COS) cells [35] and yeast [36] and then, very importantly, achieved in bacterial systems in the early 1990s [37–39]. The high-yield expression methods were important for the crystallization of human P450s, which was done primarily by Johnson and his associates following their success with a rabbit subfamily 2C P450 [40, 41]. Today, the three-dimensional structures of at least 21 human P450s have been determined (Table 9.1).

Recombinant DNA technology allowed for insight into the regulation of human P450 genes and also for the analysis of single nucleotide variations (SNVs), which could sometimes be associated with altered drug or steroid metabolism

Table 9.1 Classification of human P450s based on major substrate class

Sterols	Xenobiotics	Fatty acids	Eicosanoids	Vitamins	Unknown
1B1[a]	1A1[a]	2J2	4F2	2R1[a]	2A7
7A1[a]	1A2[a]	2U1	4F3	24A1[c]	2S1
7B1	2A6[a]	4A11	4F8	26A1	2W1
8B1	2A13[a]	4B1	5A1	26B1	4A22
11A1[a]	2B6[a]	4F11	8A1[a]	26C1	4F22
11B1	2C8[a]	4F12		27B1	4X1
11B2*	2C9[a]	4V2		27C1	4Z1
17A1[a]	2C18				20A1
19A1[a]	2C19[a]				
21A2[b]	2D6[a]				
27A1	2E1[a]				
39A1	2F1				
46A1[a]	3A4[a]				
51A1[a]	3A5				
	3A7				
	3A43				

[a] X-ray crystal structure(s) reported (for human enzyme)
[b] Bovine X-ray crystal structure reported [42]
[c] Rat X-ray crystal structure reported [43]

Table 9.2 Human P450 locations and marker reactions

P450	Tissue sites	Subcellular localization[a]	Typical reaction[b]
1A1	Lung, several extrahepatic sites	ER	Benzo[*a*]pyrene 3-hydroxylation
1A2	Liver	ER	Caffeine N^3-demethylation
1B1	Several extrahepatic sites	ER[c]	17β-Estradiol 4-hydroxylation
2A6	Liver, lung, and several extrahepatic sites	ER	Coumarin 7-hydroxylation
2A7		ER	
2A13	Nasal tissue	ER	Activation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)
2B6	Liver, lung	ER	(*S*)-Mephenytoin *N*-demethylation
2C8	Liver	ER[c]	Taxol 6α-hydroxylation
2C9	Liver	ER	Tolbutamide methyl hydroxylation
2C18	Liver	ER	
2C19	Liver	ER	(*S*)-Mephenytoin 4′-hydroxylation
2D6	Liver	ER[c]	Debrisoquine 4-hydroxylation
2E1	Liver, lung, other tissues	ER[c]	Chlorzoxazone 6-hydroxylation
2F1	Lung	ER	3-Methylindole activation
2J2	Lung	ER	Arachidonic acid oxidations
2R1	Liver	ER	Retinoic acid oxidation
2S1	Lung	ER	(several drug reductions)
2U1	Thymus, brain	ER	Arachidonic acid oxidation
2W1	Tumors	ER	
3A4	Liver, small intestine	ER[c]	Testosterone 6β-hydroxylation
3A5	Liver, lung	ER	Testosterone 6β-hydroxylation
3A7	Fetal liver	ER	Testosterone 6β-hydroxylation
3A43	Brain, liver	ER	
4A11	Liver, kidney	ER	Fatty acid ω-hydroxylation
4A22	Liver, kidney	ER	
4B1	Lung	ER	Lauric acid ω-hydroxylation
4F2	Liver	ER	Leukotriene B_4 ω-hydroxylation
4F3	Neutrophils	ER	Leukotriene B_4 ω-hydroxylation
4F8	Seminal vesicles	ER	Prostaglandin ω-2 hydroxylation
4F11	Liver	ER	Fatty acid ω-hydroxylation
4F12	Liver	ER	Arachidonic acid ω-,ω-1 hydroxylation
4F22	Liver	ER	Vitamin K ω-hydroxylation
4V2	Eye	ER	Fatty acid ω-hydroxylation
4X1	Liver, brain	ER	
4Z1	Breast cancer	ER	
5A1	Platelets	ER	Thromboxane A_2 synthase reaction
7A1	Liver	ER	Cholesterol 7α-hydroxylation
7B1	Brain	ER	DHEA 7α-hydroxylation
8A1	Aorta, others	ER	Prostacyclin synthase reaction
8B1	Liver	ER	7α-Hydroxycholesterol 12-hydroxylation
11A1	Adrenals, other steroidogenic tissues	Mit	Cholesterol side-chain cleavage
11B1	Adrenals	Mit	11-Deoxycortisol 11-hydroxylation
11B2	Adrenals	Mit	Corticosterone 18-hydroxylation
17A1	Steroidogenic tissues	ER	Pregnenolone 17α-hydroxylation
19A1	Steroidogenic tissues, adipose	ER	Androgen aromatization
20A1	Liver, other tissues	ER	

Table 9.2 (continued)

P450	Tissue sites	Subcellular localization[a]	Typical reaction[b]
21A2	Steroidogenic tissues	ER	17α-Hydroxyprogesterone 21-hydroxylation
24A1	Kidney	Mit	25-Hydroxyvitamin D_3 24-hydroxylation
26A1	Several	ER	Retinoic acid 4-hydroxylation
26B1	Brain	ER	Retinoic acid 4-hydroxylation
26C1		ER	Retinoic acid 4-,18-hydroxylation
27A1	Liver	Mit	Sterol 27-hydroxylation
27B1	Kidney	Mit	Vitamin D_3 1-hydoxylation
27C1	Liver	Mit	
39A1	Liver, other tissues	ER	24-Hydroxycholesterol 7-hydroxylation
46A1	Brain	ER	Cholesterol 24-hydroxylation
51A1	Liver, testes	ER	Lanosterol 14α-demethylation

DHEA dehydroepiandrosterone
[a] *ER* endoplasmic reticulum (microsomal), *Mit* mitochondria
[b] If known
[c] Mainly ER, some detected in mitochondria

(http://www.cypalleles.ki.se). (The term "variations" will be used here, in that "polymorphism" is usually defined as an occurrence at a ≥1% frequency [44], and many of the cases to be described here are observed at lower frequencies.) Ultimately, the availability of the human genome nucleotide sequence led to the discovery of more P450 genes. Most of the P450s listed in the "unknown" substrate column in Table 9.1 were found in this way, on the basis of the signature sequence surrounding the Cys residue that serves as the axial heme ligand. Very importantly, the number of P450 genes was set at 57 (Tables 9.1 and 9.2), thereby closing old debates on the subject [45, 46].

As mentioned earlier, the history of P450 research can be traced to early studies on the metabolism of drugs, carcinogens, and steroids. Application in these areas was remarkable in the period 1985 to present, and each area will be treated separately. Overall, the P450 field can be considered a model for how basic research can lead to important developments for human medicine. Defects in several of the P450s have been linked to serious human diseases (Table 9.3).

9.2 Relevance of P450s in Drug Metabolism

P450s are the major enzymes involved in human drug metabolism (Fig. 9.1). In looking at the fraction of the number of (small molecule) drugs processed by enzymes (Fig. 9.1a), P450s account for ~75%. Constructing a figure of this type can be somewhat misleading in that the contribution of each P450 is more difficult to evaluate in vivo than in vitro (for an earlier tabulation, see [51]). The large contributions of P450s 3A(4) and 2C9 are driven to a large extent by the high levels of expression of these two enzymes in human liver (and small intestine) and to their broad substrate specificity (Figs. 9.2 and 9.3). The charts do not necessarily reflect all drugs currently in development. A current tendency has been the development of larger molecules as drug candidates, in order to achieve target specificity and affinity, and a general axiom is that these are more readily accommodated by P450s 3A4 and 2C9. In recent years, pharmaceutical companies have tried to avoid developing drug candidates that are substrates (or inhibitors) for the highly variant

Table 9.3 Some diseases associated with defects in *CYP* genes [47, 48]

Gene	Disorder
CYP1B1	Primary congenital glaucoma (buphthalmos)
CYP2R1	Rickets
CYP4A	Defects in salt metabolism, water balance leading to arterial hypertension
CYP4F22	Ichthyosis
CYP4V2	Bietti's crystalline dystrophy
CYP5A1, 8A1	Defects leading to clotting and inflammatory disorders, coronary artery disease, and pulmonary hypertension
CYP7A1	Hypercholesterolemia
CYP7B1	Severe hyperoxysterolemia and neonatal liver disease
CYP11A1	Lipoid adrenal hyperplasia; occasional congenital adrenal hyperplasia (CAH)
CYP11B1	Occasional CAH
CYP11B2	Corticosterone methyloxidase deficiency type I, or type II; occasional CAH
CYP11B1, 11B2	Chimeric enzymes causing glucocorticoid-remediable aldosteronism; occasional CAH
CYP17A1	Mineralocorticoid excess syndromes, glucocorticoid and sex hormone deficiencies; association with increased risk of prostate cancer and benign prostatic hypertrophy; occasional CAH
CYP19A1	Loss of function: virilization of females, hypervirilization of males, occasional CAH; gain of function: gynecomastia in young males
CYP21A2	>90% of all CAH
CYP24A1[a]	Hypervitaminosis D
CYP27A1	Cerebrotendinous xanthomatosis
CYP27B1	Vitamin D-dependent rickets type I
CYP46A1[a]	Learning disability

[a] Evidence of disease in animal models but not yet in clinical studies

P450s 2D6 and 2C19. With all of these caveats in hand, the allocation of the P450s in the chart in Fig. 9.1b is probably a good estimate and will not change considerably in the near future. However, a point to be made here is that the metabolism of many drugs is a function not only of P450s but also other enzymes and, as recognized more in recent years, transporters that alter the concentrations of drugs within cells. A discussion of drug transporters is outside the scope of this chapter, and the reader is referred elsewhere [57–59].

The subjects of P450 regulation and variation have already been mentioned and will be treated again with individual P450s. At this point, some general practical considerations are discussed. If one considers the total concentration of P450 in liver samples from different healthy individuals (on a milligram protein basis), most individuals fall within a range of ~threefold [28]. However, when individual "drug-metabolizing" P450s (e.g., families 1, 2, 3) are considered, the variation is considerable, with five- to tenfold being common and 40-fold not unusual, e.g., P450 1A2 [60]. With P450 1A2, a similar variability (40-fold) is seen in in vivo caffeine pharmacokinetics [61]. With some enzymes, the variability in the same in vivo pharmacokinetic parameters can be 10^4-fold (Fig. 9.4).

Two examples of studies of the variability among individuals are presented in Fig. 9.5 (Caucasians) and Fig. 9.6 (Caucasians and Japanese). Gender has not been shown to have a major influence on levels of expression of the major xenobiotic-metabolizing P450s [64] (with a German P450 3A4 study seemingly unusual [65]), and inter-gender pharmacokinetic differences are probably due to other influences of bioavailability [66]. Racial differences exist due to allelic variations, which may influence either levels of expression or the inherent catalytic activity of the P450s (e.g., P450 2D6 [67]). Some apparent racial differences are seen here (Fig. 9.6) and have also been reported in in vivo studies (e.g., P450 3A4 [68], P450 2E1 [69]). Controlling diets is an issue in many in vivo studies of this type, and in vitro studies can also be affected. In general, the differences in activities of a given P450 between races are much less than within a race (e.g., Fig. 9.6). Finally, the

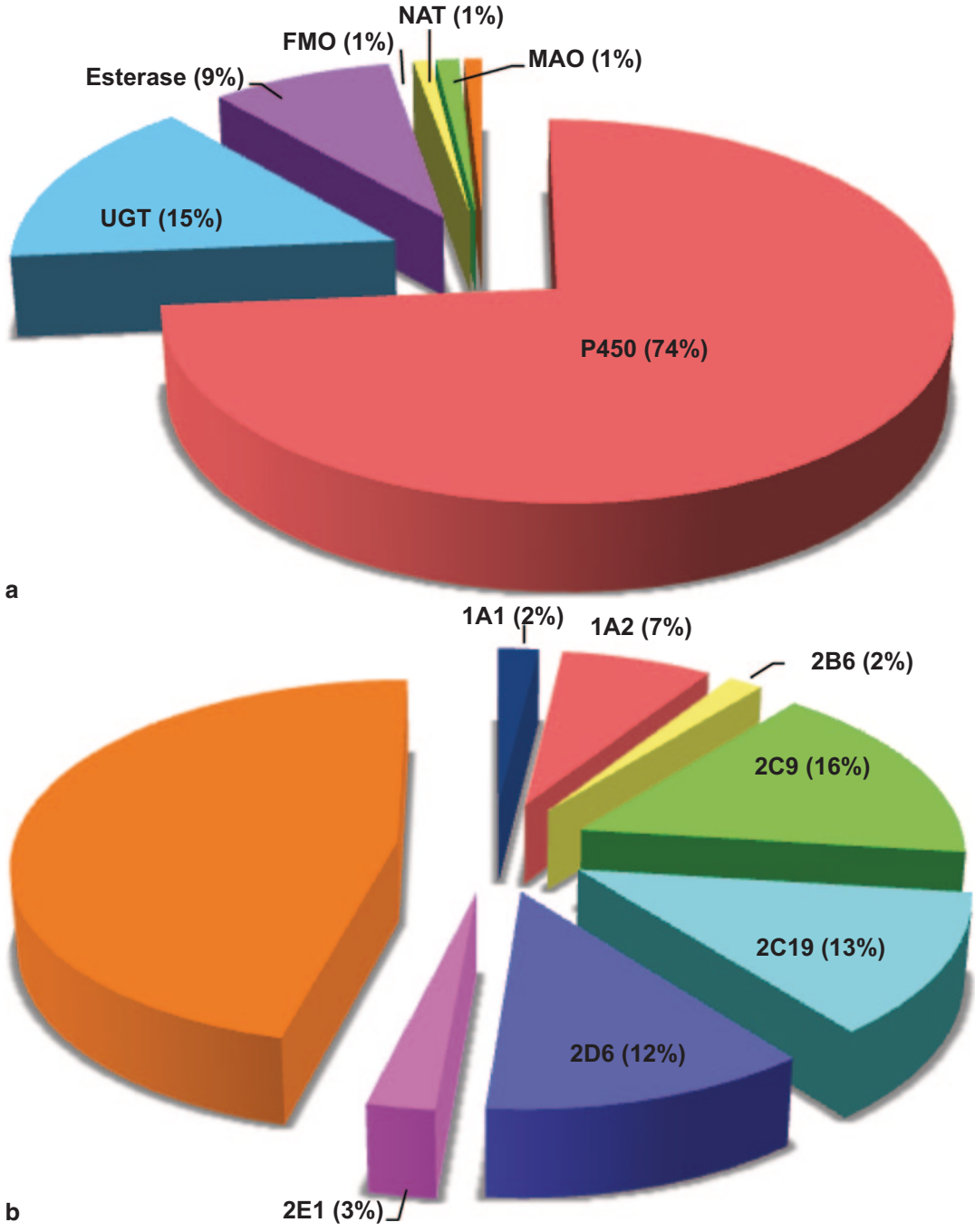

Fig. 9.1 The enzymes of drug metabolism. **a** Contributions of different enzymes to drug metabolism. **b** Contributions of individual human P450 enzymes to (P450) drug metabolism [49] (see also [50]). *UGT* UDG glucuronosyl transferase, *FMO* flavin-containing monoxygenase, *NAT N*-acetyltransferase, *MAO* monoamine oxidase

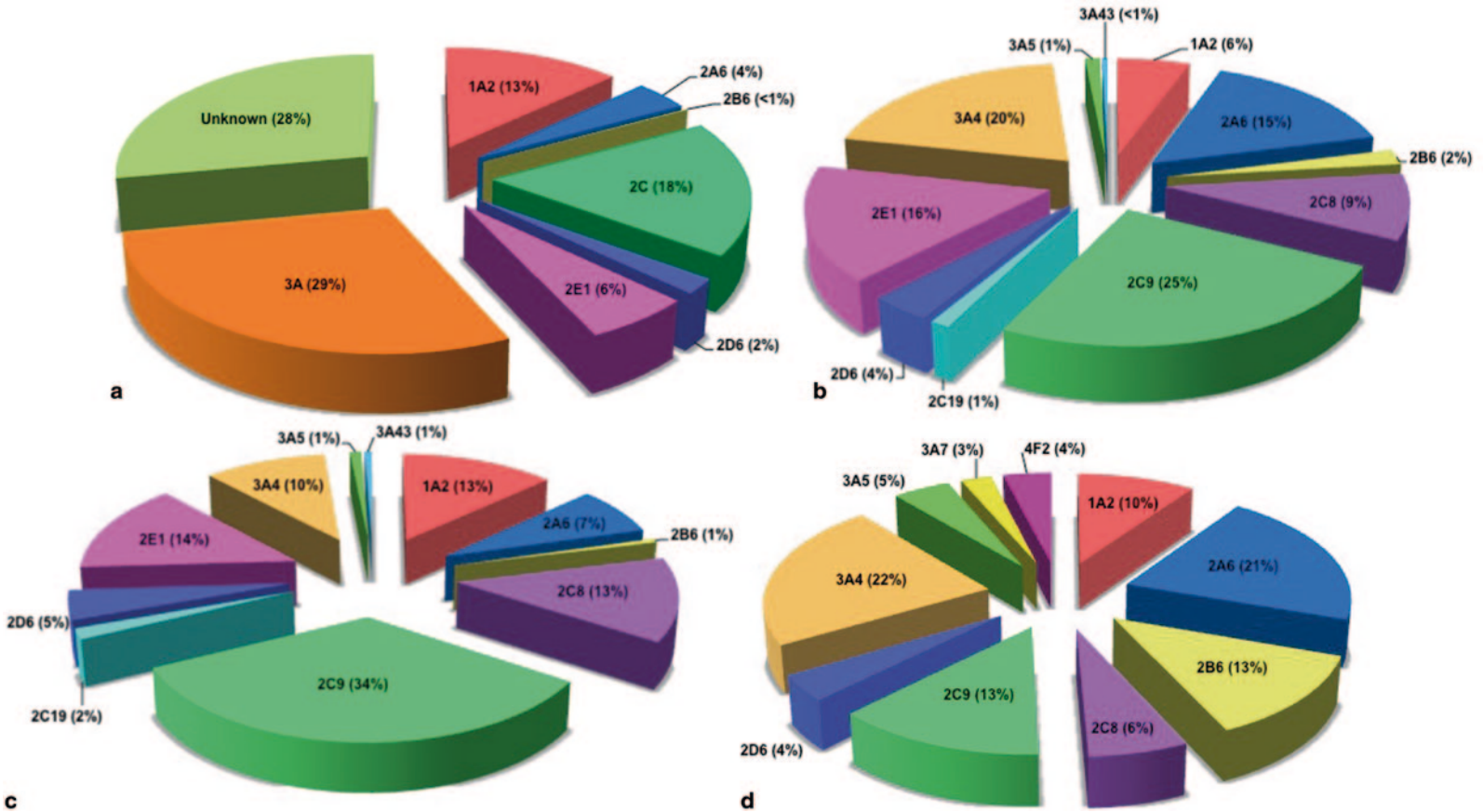

Fig. 9.2 Relative concentrations of P450 in human liver microsomes. **a** P450s in 60 liver samples were estimated using immunochemical methods (electrophoresis/immunoblotting) [52]. Because of cross-reactivity, the individual P450s in subfamilies are not distinguished. The "unknown" fraction is the difference between the sum of the immunochemically determined forms and the total amount, calculated from $Fe^{2+}{\cdot}CO$ versus Fe^{2+} difference spectroscopy [53]. **b–d** Estimates were made using liquid chromatography–mass spectrometry (LC–MS) proteomic analysis with heavy-atom peptides. **b** Results of an analysis of 50 pooled human liver samples (XenoTech, HLM610 preparation) [54]. **c** Results reported in the same reference as Part *B* [54] as means from analysis of ten individual human samples. **d** Analysis of a pooled set of 23 human liver samples by another laboratory [55]

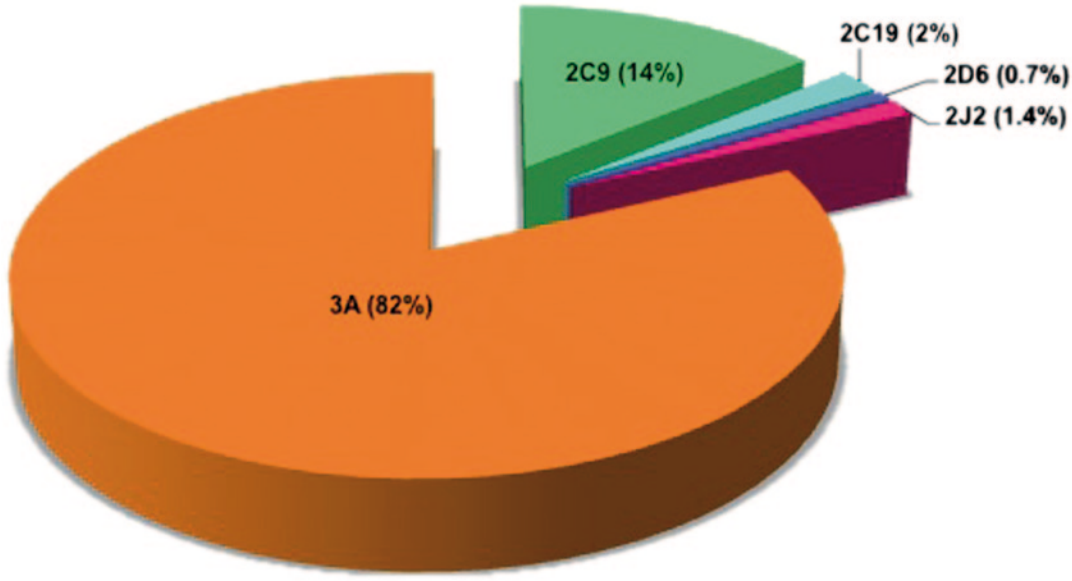

Fig. 9.3 Relative concentrations of individual P450s in human intestine (determined immunochemically) [56]. *3A* indicates all subfamily 3A P450s

point should be made that the levels of the P450s involved in steroid hormone metabolism (e.g., first column of Table 9.1) vary considerably less than do the xenobiotic-metabolizing P450s (families 1, 2, 3), probably due to their well-defined roles in regulation of physiological processes.

Many chemicals are capable of inducing P450s, as clearly demonstrated in animals and with cell culture systems [70]. In vivo induction experiments with humans are not as readily done as with animals, but ample evidence for P450 induction is available, going back to the barbiturate observations of Remmer in the 1950s [2]. A short list of some established P450 inducers is presented in Table 9.4. This list is rather conservative in that only information is included from studies in which in vivo evidence has been obtained. Much of the studies have involved pharmacokinetics, but some "moderately invasive" studies have involved direct measurement of proteins, messenger RNA (mRNA), or enzyme activities in peripheral blood cells or small intestinal biopsies; liver biopsy data are rare. Table 9.4 could probably be expanded considerably if all information from in vitro studies were included, e.g., P450s 1B1 and 2S1 are probably inducible by aryl hydrocarbon receptor (AhR) ligands [71, 72]. The major problem in demonstrating human P450 induction in vivo is the lack of diagnostic pharmacokinetic parameters for many of P450s.

The clinical influence of differences in P450 activity can be rationalized using the scheme of Fig. 9.7. A list of major drug substrates of each human P450, from the Indiana University website (http://medicine.iupui.edu/clinpharm/ddis/maintable/), is presented in Tables 9.5, 9.6, and 9.7. This is intended to be useful but not comprehensive, and of course more drugs will continue to be added. Drug doses are generally developed with the extensive metabolizers (EMs) as the general population of major interest, or at least this was the emphasis in the past. The plasma concentration rises to a peak ($C_{p,max}$) following the first dose and then decreases to a lower level prior to the next dose. With subsequent doses, the plasma concentration remains within this region and yields the desired pharmacological effect. Without prior knowledge about a problem with this drug, the poor metabolizer (PM; lower panel of Fig. 9.7) would be administered the same doses. Very limited metabolism would occur between doses, and the plasma concentration of the drug (and presumably the concentration of the drug in the target tissue) will rise to an unexpectedly high

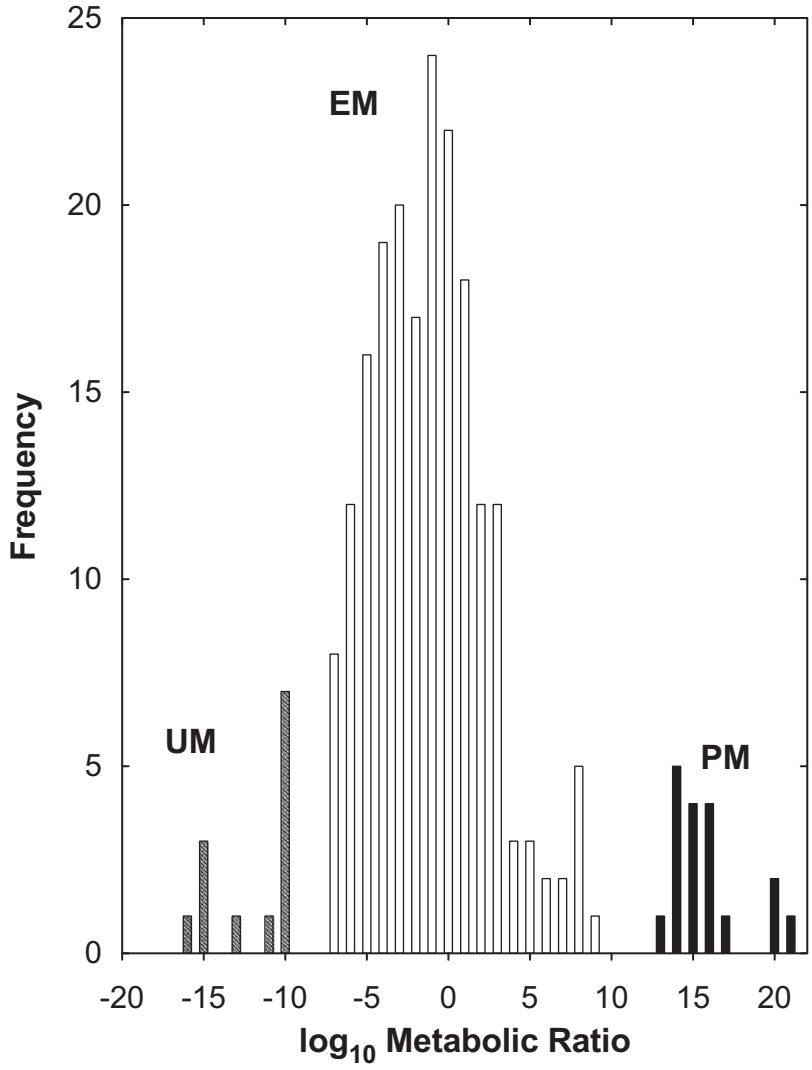

Fig. 9.4 Frequency distribution histogram of (in vivo) debrisoquine 4-hydroxylation in a Caucasian population [62]. The metabolic ratio is the ratio of debrisoquine/4-hydroxydebrisoquine in the urine of individuals who were administered debrisoquine (10-mg free base) 8 h previously. The groups are designated *PM* (poor metabolizers,) and *EM* (extensive metabolizers,). The group labeled *UM* (ultra-metabolizer) is from retrospective research [63] and probably represents gene duplication. (With kind permission from Springer Science + Business Media: [149], Fig. 10.5)

level, with an attendant increase in the area under the curve (AUC). The simplest effect would be an exaggerated (and probably undesirable) pharmacological response. Sometimes there is a situation in which metabolism is more rapid than expected in the typical patient (Fig. 9.4), e.g., due to gene amplification or enzyme induction. In this case, $C_{p,max}$ and AUC would be smaller than in the case of the EM (Fig. 9.7, upper panel), and decreased drug efficacy would be expected.

Some practical situations follow and can be addressed in the context of our current general knowledge of substrates, inducers, and inhibitors (Fig. 9.8, Tables 9.5, 9.6, and 9.7). With regard to polymorphisms and other variations, several are known that can render some drugs impracti-

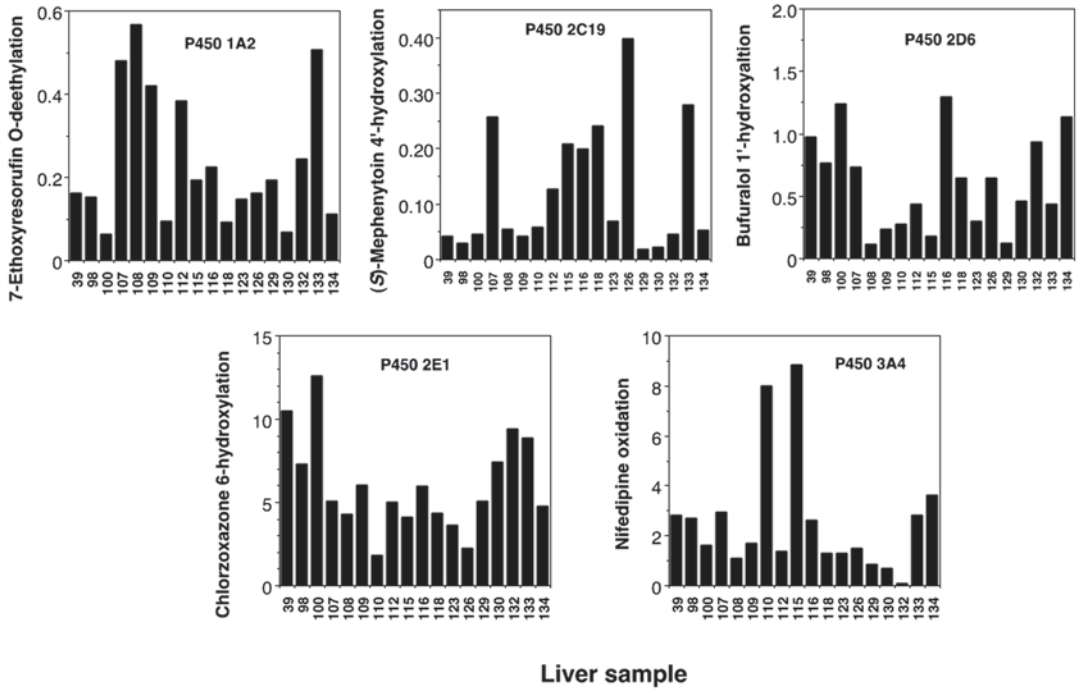

Fig. 9.5 Variation in levels of five P450s in 18 human liver samples. Individual P450s and catalytic activities are indicated on each chart [2768]. Sample number refers to a code from this laboratory. (With kind permission from Springer Science + Business Media: [149], Fig. 10.1)

cal due to toxicity (e.g., perhexiline, leading to peripheral neuropathy due to lack of metabolism by P450 2D6 [75]) or can alter the recommended dose (e.g., warfarin/P450 2C9 [76–78] and omeprazole/P450 2C19 [79, 80]). Perhaps surprisingly, no deaths have been documented to date due to PM phenotypes (to the author's knowledge and in a discussion with Robert Smith), although it is possible that these have occurred but not recognized. However, a death of a nursing infant occurred because the mother was an ultrarapid metabolizer (Fig. 9.4) and the codeine she used resulted in an overdose of the P450 2D6 product morphine in her breast milk [81].

Drug interactions are a serious problem, and pharmacokinetic interactions have several molecular bases. One is enzyme induction, which usually results in decreased bioavailability. The decreased bioavailability of a drug can be the result of induction by that same drug or by another drug. A classic example is the decreased bioavailability of the oral contraceptive 17α-ethinylestradiol following treatment of individuals with rifampicin, barbiturates, or St. John's wort, leading to P450 3A4 induction [26, 82, 83]. Another aspect of drug–drug interactions involves P450 inhibition. The inhibition can be of a competitive nature, i.e., two substrates competing for a limiting amount of a P450 or a bona fide inhibitor (no enzymatic transformation) competing with substrates. An example here is the antihistamine terfenadine, the metabolism of which is inhibited by the P450 3A4 inhibitors erythromycin and ketoconazole. Another major type of P450 inhibition is "mechanism-based" (or "suicide") inactivation, in which oxidation of a substrate destroys the P450 [84, 85]. An example here is the inactivation of P450 3A4 by bergamottin and other flavones found in grapefruit juice [86–89].

In the above cases, the effects have been discussed only in terms of altered bioavailability; i.e., with increased clearance of 17α-ethinylestradiol, unexpected menstrual bleeding

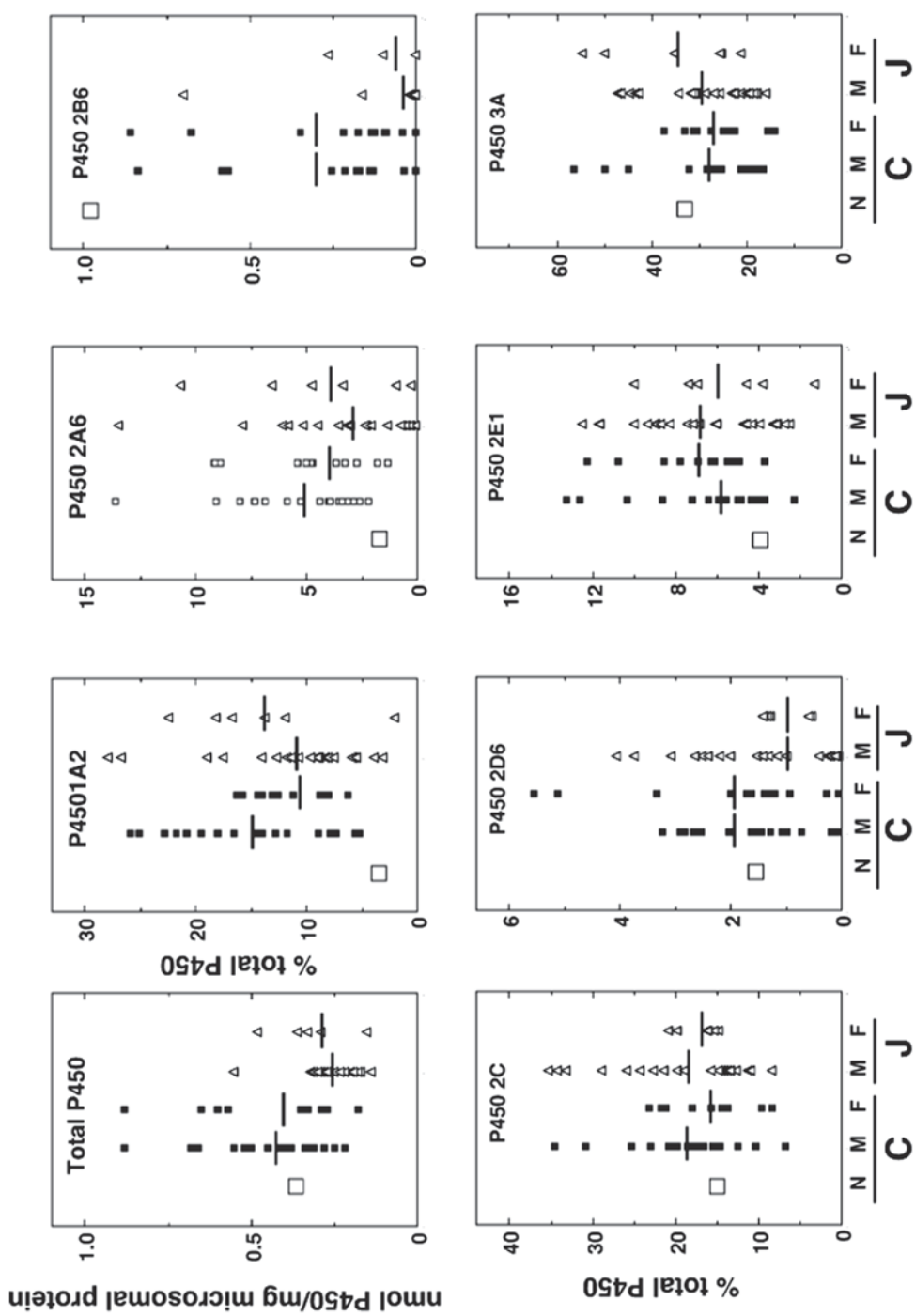

Fig. 9.6 Comparison of some immunochemically determined levels of individual P450s and catalytic activities in human liver microsomes. Results from samples obtained from Caucasian (*C*) and Japanese (*J*) males (*M*), females (*F*), and a single neonatal sample (*N*) are shown [52]. The vertical axis is nmol P450/mg protein in the *Total P450* chart and % of total P450 in all other cases. (With kind permission from Springer Science + Business Media: [149], Fig. 10.2)

Table 9.4 Some major inducers of human P450 enzymes

Class of inducers	Some sources	Example	Subfamily P450s induced[a]
AhR ligands	Tobacco, broiled meat, accidental exposures to pollutants	Polychlorinated biphenyls	1A1, 1A2
Barbiturates and similar compounds	Drugs, some polyhalogenated biphenyls, DDT	Diphenylhydantoin	2C, 3A
PXR ligands	Some steroids and antibiotics, other drugs	Rifampicin	3A
P450 2E1 inducers	Ethanol, isoniazid	Ethanol	2E1

AhR aryl hydrocarbon receptor, *DDT* dichlorodiphenyltrichloroethane, *PXR* pregnane X receptor
[a] Based on in vivo responses

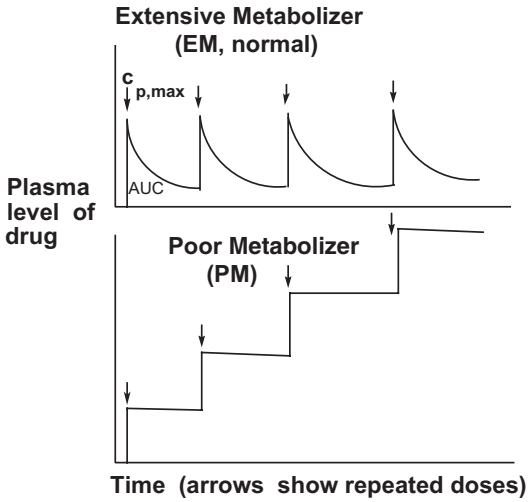

Fig. 9.7 Significance of low metabolism of a drug by P450s (or other enzymes). A "typical" pattern is seen in the upper panel (*EM*), where the plasma level of the drug is maintained in a certain range when a particular repetitive dose is prescribed. Unusually, slow metabolism (lower panel, *PM*) results in an elevated plasma level of the drug. $C_{p,max}$ = maximum plasma concentration, *AUC* area under the curve. (Reproduced with kind permission from Springer Science + Business Media: [149], Fig. 10.8)

and pregnancies have resulted [83, 90, 91]. Some of the drug interaction problems can be more complex, even when the analysis is restricted to pharmacokinetic aspects. For instance, in the example mentioned above, terfenadine can be considered a prodrug [92]; in most individuals, the P450 oxidation (followed by further oxidation) yields fexofenadine, the circulating (and active) form of the drug. Low levels of P450 3A4 activity (due to inhibition or other reasons) [93, 94] cause the accumulation of the parent (prodrug) terfenadine to toxic levels that can cause arrhythmias [92, 95]. Another possibility is that blocking a primary route of metabolism of a drug may favor secondary pathways that lead to toxicity, e.g., blocking phenacetin *O*-deethylation (P450 1A2) can lead to deacetylation, *N*-oxygenation, and methemoglobinemia [96]. Although a good example is not available, it is possible that blocking the oxidation of one drug by a P450 could cause it to accumulate and behave as an inhibitor towards another. A potential example would be decreasing the P450 3A4-catalyzed oxidation of quinidine and having the accumulated drug inhibit P450 2D6 [97]. P450 induction could result not only in decreased oral availability but also in the enhanced bioactivation of chemicals. This is a general concern with potential carcinogens, as discussed in the next section of this chapter, and one of the reasons why regulatory agencies have concern about P450 1A inducers.

In the process of drug development, there are several guiding principles to dealing with P450 metabolism, aside from details of each specific case: (1) use of in vitro screening to eliminate compounds that will have poor bioavailability (i.e., rapid in vitro oxidation); (2) use of in vitro screens to avoid obvious problems of toxicity, induction, and inhibition; (3) searching for drug candidates in which the metabolism is the result of several different enzymes and not dependent upon a single one, particularly a highly variable P450 (or other enzyme); and (4) use of in vivo human studies to address in vitro predictions as early as possible.

Table 9.5 Human P450 drug interactions—substrates. (Reproduced from http://medicine.iupui.edu/clinpharm/ddis/main-table/ with permission from the Indiana University Division of Clinical Pharmacology)

1A2	2B6	2C8	2C9	2C19	2D6	2E1	3A4,5,7
Clozapine	Bupropion cyclophosphamide	Paclitaxel	**NSAIDs** Diclofenac	**PPIs** Lansoprazole	**Beta-blockers**	**Anesthetics** Enflurane	**Macrolide antibiotics**
Cyclobenzaprine		Torsemide					
Duloxetine		Amodiaquine	Ibuprofen	Omeprazole	(*S*)-metoprolol	Halothane	Clarithromycin
Fluvoxamine	Efavirenz	Cerivastatin	Piroxicam	Pantoprazole	Propafenone	Isoflurane	Erythromycin
Haloperidol	Ifosfamide	Repaglinide		Rabeprazole	Timolol	Methoxyflurane	(not 3A4)
Imipramine	Methadone		**Oral hypoglycemics**			Sevoflurane	NOT
Mexiletine				**Antiepileptics**	**Antidepressants**		Azithromycin
Nabumetone			Tolbutamide		Amitriptyline	**Others**	Telithromycin
Naproxen			Glipizide	Diazepam	Clomipramine	Acetaminophen	
Benzene							
Olanzapine			**Angiotensin II blockers**	Phenytoin	Desipramine	Chlorzoxazone	**Anti-arrhythmics**
Riluzole				Phenobarbitone	Imipramine	Ethanol	
Tacrine			Losartin		Paroxetine	*N,N*-dimethyl-formamide	quinidine→3-OH
Theophyline			Irbesartan	**Others**			
Tizanidine				Amitriptyline	**Antipsychotics**	Theophyline	
Triamterene			**Others**	Clomipramine	Haloperidol		**Benzodiazepines**
Zileuton			Celecoxib	Clopidogrel	Risperidone		Alprazolam
Zolmitriptan			Fluvastatin	Cyclophosphamide	Thioridazine		Diazepam
			Naproxen				Midazolam
			Phenytoin	Progesterone	**Others**		Triazolam
			Rosiglitazone		Aripiprazole		
			Sulfamethoxazole		Codeine		**Immune modulators**
			Tamoxifen		Dextromethorphan		
			Tolbutamide		Duloxetine		Cyclosporine
			Torsemide		Flecainide		Tacrolimus
			Warfarin		Mexiletine		(FK506)
					Ondansetron		
					Tamoxifen		**HIV antivirals**
					Tramadol		Indinavir
					Venlafaxine		Ritonavir
							Saquinavir

Table 9.5 (continued)

1A2	2B6	2C8	2C9	2C19	2D6	2E1	3A4,5,7
							Prokinetics
							Cisapride
							Antihistamines
							Astemizole
							Chlorpheniramine
							Calcium channel blockers
							Amiodipine
							Diltiazem
							Felodipine
							Nifedipine
							Nisoldipine
							Nitrendipine
							Verapamil
							HMG-CoA reductase inhibitors
							Atorvastatin
							Lovastatin
							Simvastatin
							Others
							Aripiprazole
							Boceprevir
							Buspirone
							Gleevec
							Haloperidol
							Methadone
							Pimozide
							Quinine
							Sildenafil
							Tamoxifen
							Telaprevir
							Trazodone
							Vincristine

NSAID nonsteroidal anti-inflammatory drug, *PPI* proton pump inhibitor, *HMG-CoA* 3-hydroxy-3-methyl-glutaryl-coenzyme A

Table 9.6 Human P450 inhibitors (http://medicine.iupui.edu/clinpharm/ddis/main-table/)

1A2	2B6	2C8	2C9	2C19	2D6	2E1	3A4,5,7
Cimetidine	Thiotepa	Gemfibrozil	Fluconazole	Esomeprazole	Bupropion	Disulfiram	**HIV antivirals**
Fluoroquinolones	Ticlopidine	Montelukast	Amiodarone	Fluoxetine	Fluoxetine		
Fluvoxamine			Isoniazid	Fluvoxamine	Paroxetine		Indinavir
Ticlopidine				Ketoconazole	Quinidine		Nelfinavir
				Lansoprazole	Duloxetine		Ritonavir
				Omeprazole	Amiodarone		
				Ticlopidine	Cimetidine		**Antimicrobials**
					Chlor-pheniramine		
							Clarithromycin
					Doxepin		
					Haloperidol		Itraconazole
					Methadone		Ketoconazole
					Mibefradil		Nefazodone
					Ritonavir		
							Others
							Erythromycin
							Grapefruit
							Juice
							Verapamil
							Suboxone
							Diltiazem
							Cimetidine
							Amiodarone
							Fluvoxamine
							Mibefradil
							Troleandomycin

Table 9.7 Human P450 inducers (http://medicine.iupui.edu/clinpharm/ddis/main-table/)

1A2	2B6	2C8	2C9	2C19	2D6	2E1	3A4,5,7
Smoking	Phenobarbital		Rifampin			Ethanol	Carbamazepine
	Phenytoin		Secobarbital			Isoniazid	Phenobarbital
	Rifampin						Phenytoin
							Pioglitazone
							Rifabutin
							Rifampin
							St. John's Wort
							Troglitazone

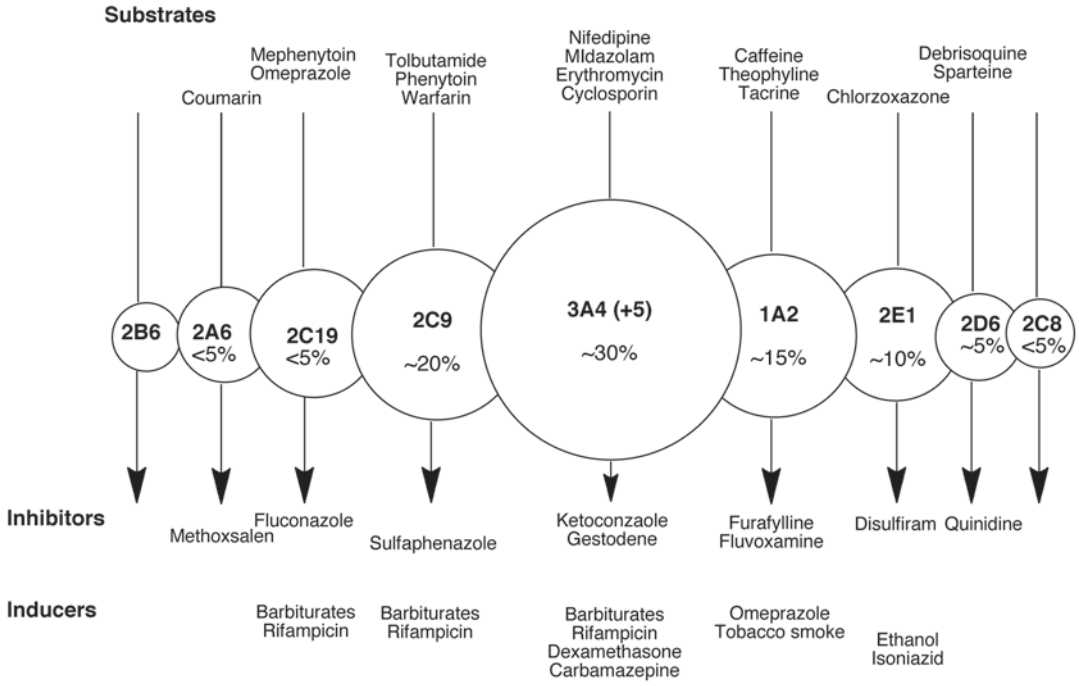

Fig. 9.8 A summary of major human P450s involved in drug metabolism, including major substrates, inhibitors, and inducers (adapted from [73, 74]. The sizes of the *circles* indicate the approximate mean percentages of the total hepatic P450 attributed to each P450 (see also Fig. 9.2). The overlap of the circles is to make the point that overlap of catalytic action is often observed, although the overlap does not necessarily refer to the indicated substrates (or inhibitors). (With kind permission from Springer Science + Business Media: [149], Fig. 10.4)

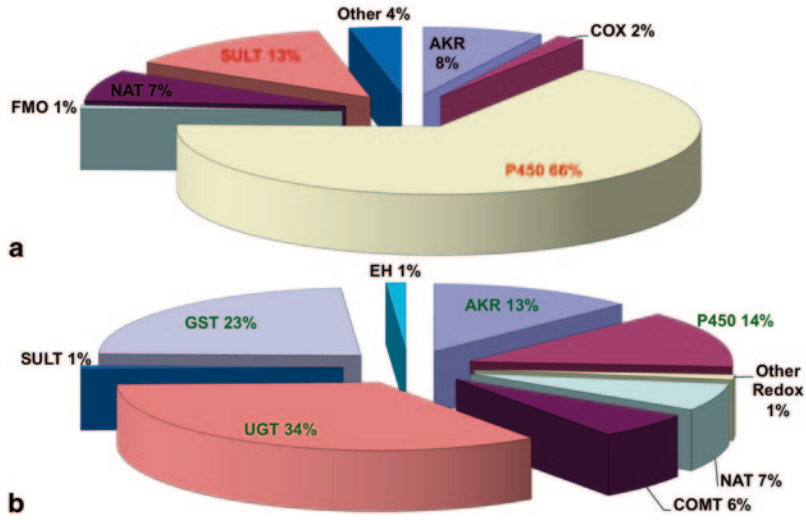

Fig. 9.9 Carcinogen metabolism by human enzymes [99]. **a** Contributions of different (human) enzyme systems to carcinogen activation. **b** Contributions of different (human) enzyme systems to carcinogen detoxication. *FMO* flavin-containing monoxygenase, *NAT* *N*-acetyltransferase, *SULT* sulfotransferase, *AKR* aldo-keto reductase, *COX* cyclooxygenase/prostaglandin synthase, *UGT* UDG glucuronosyl transferase , GST glutathione transferase, COMT catechol O-methyl transferase.

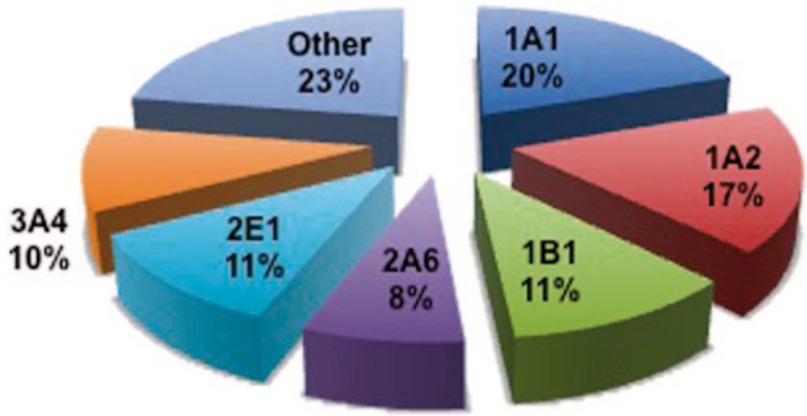

Fig. 9.10 Contributions of individual human P450s to the P450 sector of carcinogen activation [99]

9.3 Relevance of P450s in Toxicology and Cancer Risk

Historically, much of the attention given to P450s has come from the interest in cancer, going back to some of the first demonstrations of oxidation and reduction reactions in the metabolism of chemical carcinogens [98] and the inducibility of P450s by carcinogens [1] (Figs. 9.9 and 9.10). The interest in P450s was also extended to chemical toxicities other than cancer with the demonstration of bioactivation of compounds such as the drug acetaminophen [100] and the insecticide parathion [101, 102]. Many studies have been done with P450 animal models, particularly using P450 inducers and inhibitors and genetically modified mice, either naturally occurring or transgenic. These studies provide strong evidence that alterations in the activities of P450s can

modify the sensitivity of mice to various chemicals. For instance, the *Ah* locus (which controls P450s 1A1, 1A2, and 1B1 as well as some conjugating enzymes) can modify the sensitivity in AhR-deficient mice, depending upon the chemical and the organ site [103]. Effects of specific P450 knockouts have been reported in transgenic mice as well, e.g., prevention of acetaminophen toxicity by deleting P450s 2E1 and 1A2 [104, 105] and of 7,12-dimethylbenz[*a*]anthracene-induced lymphomas by deleting P450 1B1 [106].

When the enzymes involved in the activation of chemical carcinogens in humans are considered, two-thirds of the reactions are catalyzed by P450 enzymes (Fig. 9.9a) [99]. Of the human P450s, six account for 77% of these reactions (Fig. 9.10). The three family 1 P450s (1A1, 1A2, 1B1) account for one half of the reactions [99]. Two other points should be made. One is that the reported distributions (Fig. 9.10) are a function of how many compounds in prominent classes have been considered. That is, P450s 1A2 and 1B1 activate many arylamines, P450s 1A1 and 1B1 activate many polycyclic hydrocarbons, P450 2A6 and 2E1 activate many *N*-nitrosamines, etc. Therefore, the pattern may change in the future as other categories are studied more. The other point is that P450s are involved in detoxication reactions. About 14% of the enzymatic detoxication reactions are done by P450s (Fig. 9.9b), including *C*-hydroxylations, reductions, and *N*-oxidations [99].

Despite the strong evidence for effects of variability of P450 on chemical toxicity and cancer risk in animals and the knowledge that human P450 levels vary considerably (Figs. 9.5, 9.6, 9.7, and 9.11), demonstrating relationships with human disease has been difficult. In the 1960s, the demonstration of the inducibility of aryl hydrocarbon hydroxylase (P450 1A1 and possibly P450 1B1) by Nebert and Gelboin [107] led to more investigations with human samples, particularly peripheral blood cells. The work of Shaw and Kellerman [108, 109] suggested that the inducibility of aryl hydrocarbon hydroxylase (now recognized as P450 1A1 and 1B1 under these conditions) is correlated with susceptibility of smokers to lung cancer. In the early work, this apparently genetic variability was trimodal. Subsequently, this phenomenon has proven difficult to study, in part due to technical difficulties in the earlier phases of the work [110]. Many of the early problems have been circumvented with the ability to measure mRNA expression and the access to DNA sequences. While evidence for correlation of P450 1A1 mRNA expression with lung cancer incidence has been obtained [111], an unresolved issue is the nature of any genetic variability. In contrast to the situation seen in mouse models [112], the allelic variations in the human AhR (which has apparently considerably lower affinity for many of the ligands of interest than the mouse receptor [113]) do not appear to account for interindividual levels of inducibility of P450 1A1 [114, 115]. Epidemiological evidence has been presented for association of lung cancer incidence with an *Msp*I polymorphism of P450 1A1 [116]. However these results, obtained in studies done with Japanese, have not been reproduced in Caucasians [117–119]. Further, the heterologously expressed human P450 1A1 allelic variant (V462I) showed only a relatively small change in oxidation of the prototypic polycyclic aromatic hydrocarbon (PAH) carcinogen benzo[*a*]pyrene [120, 121]. A possible explanation to the quandary comes from the report that P450 1B1, not P450 1A1, is the major P450 responsible for the aryl hydrocarbon hydroxylation activity in lymphocytes and that it is P450 1B1 expression that shows the classic trimodality, not P450 1A1 [122].

Today the search for roles of a particular P450 in human disease follows a route similar to that just discussed for P450 1A1, i.e., the identification of SNVs (see earlier note about difference between variations and polymorphisms, *vide supra*) is a basis for epidemiological associations with various maladies. This approach is commonly applied to the possible roles of P450s in cancers at various organ sites. The positive aspects of this strategy are that we have an extensive knowledge base of allelic variations of P450s (e.g., http://www.imm.ki.se/cypalleles/), sophisticated and very sensitive biological tools,

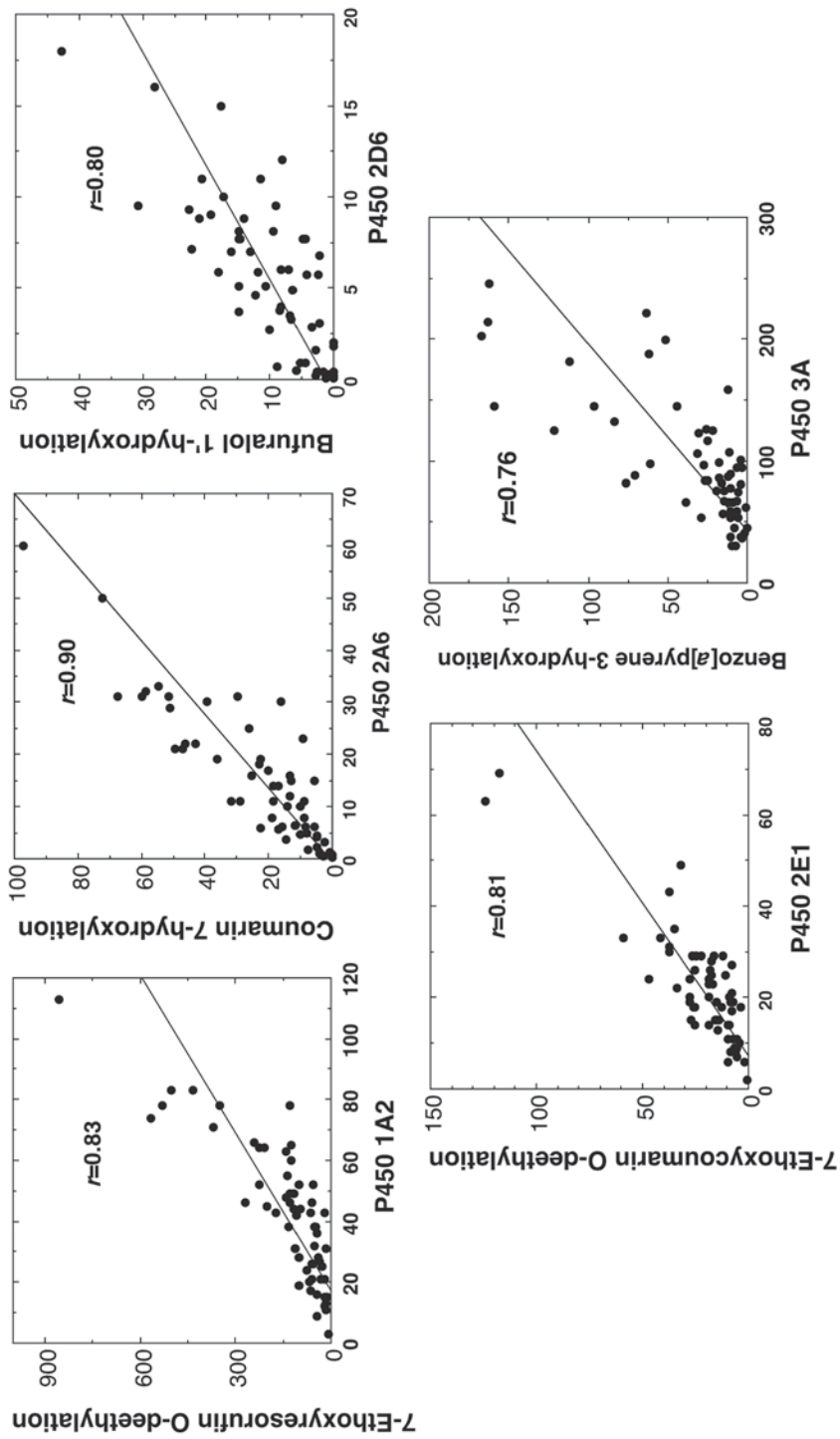

Fig. 9.11 Correlation of catalytic activities with immunochemically determined levels of five P450s in human liver microsomes. The correlation coefficients (r) were determined using linear regression analysis [52]. (With kind permission from Springer Science + Business Media: [149], Fig. 10.7)

and the potential to noninvasively analyze large populations, at least in the case of some diseases and P450s. On the negative side, the ability to rapidly screen for associations without serious consideration of present or past chemical exposure levels has led to many studies with little or only marginal biological plausibility. Many association studies have been difficult to repeat. An example in point is the reported association of attenuated lung cancer risk (of smokers) with the P450 2D6 PM phenotype. Although the initial reports were quite exciting [123], subsequent studies yielded variable results and meta-analysis has not supported an association [124]; no real experimental support for a biological association was ever found [125]. A review by Vineis [126] concludes that the risks of cancer due to genetics are considerably less than those associated with smoking or some other environmental factors.

What associations of P450 have been adequately demonstrated? The list below is short and not intended to necessarily be totally inclusive but emphasizes some of the more positive associations found to date. (The absence of several of the steroid-oxidizing P450s is known to be debilitating, but these are not treated here (Table 9.3); see the sections on individual P450s and reference [47].) The possible association between P450 1A1 and lung cancer has already been discussed above; a confounding factor may be expression of P450 1B1. Truncation of P450 1B1 is associated with glaucoma, for unknown reasons [127]; this defect has also been seen in P450 1B1-knockout mice, but the molecular basis is not known [128]. Allelic variants in P450 1B1 do not appear to have major effects in the oxidation of carcinogens [129]; some differences in cancer risk have been reported in the epidemiology literature [130, 131]. P450 1A2 activity has been reported to be associated with colon cancer incidence, when the factors of *N*-acetyltransferase and well-done meat intake are considered [132]; an association has plausibility in the activation of heterocyclic amines by P450 1A2 [31]. One of the stronger associations reported to date involves that of P450 2A6 with lung cancer; the association is driven by the data obtained with individuals with the gene deletion [133]. A relationship has plausibility in the demonstrated ability of P450 2A6 to activate *N*-nitrosamines (Table 9.8) and possibly in the decreased smoke intake of null-type individuals due to impaired metabolism of nicotine [134] (see Sect. 7.4.7, *vide infra*). Although many epidemiological studies have been done with SNVs of P450 2E1, any putative changes in P450 2E1 phenotype have not been validated with in vivo assays and must be considered suspect [135].

In the process of drug development, the induction of P450 family 1 and P450 2B enzymes (in animals or in human cell or reporter assays) has often been considered an issue for potential toxicity [136, 137]. The concern about induction is that the rodents may be likely to develop liver or other tumors in cancer bioassays with these compounds, and any association between these inductions and human cancer is not established; e.g., epileptics with long-term exposure to barbiturates and hydantoins have not been found to have more cancer [138]. Likewise, the induction of subfamily 4A P450s is an indicator of peroxisomal proliferation, a phenomenon associated with rodent liver tumors but probably not human [139]. Thus, induction of rodent P450s has been shown to be a means of identifying types of potential rodent toxicity [140], some of which may be relevant to humans, but should not be used as evidence for adverse roles of these agents in humans. Transgenic studies with "humanized" mouse models have provided some insight into more appropriate risk assessment [141, 142].

9.4 Relevance of P450s in Endocrinology

Another area that has driven the P450 field is steroid metabolism (Fig. 9.12). As the structures of the important steroids were elucidated in the first half of the twentieth century, it became apparent that the metabolic pathways linking these were dominated by oxidation and reduction. Subsequently, roles of P450s were discovered in the hydroxylations and even more complex oxidations involving C–C bond scissions. One of the first P450 reactions demonstrated was the steroid

Table 9.8 Some human P450 enzymes involved in the activation of carcinogens [30, 99]. (See also Table 9.10 and Fig. 9.10).

P450 1A1, 1B1	P450 1A2, 1B1	P450 2A6, 2A13	P450 2E1	P450 3A4
Benzo[*a*]pyrene and other polycyclic hydrocarbons 2-Amino-1-methyl-6-phenylimidazo-4,5-*b*]pyridine (PhIP)	PhIP 2-Amino-6-methyl-dipyrido[1,2-*a*:3,2'-*d*]-imidazole (Glu P-1) 2-Aminodipyrido-[1,2-*a*:3,2'-*d*]imidazole (Glu P-2) 2-Amino-3-methylimidazo-[4,5-*f*] quinoline (IQ) 2-Amino-3,5-dimethyl-imidazo[4,5-*f*] quinoline (MeIQ) 2-Amino-3,8-dimethyl-imidazo[4,5-*f*] quinoline (MeIQx) 3-Amino-1-methyl-5*H*-pyrido[4,3-*b*] indole (Trp P-2) 4-Aminobiphenyl 2-Naphthylamine 2-Aminofluorene 2-Acetylaminofluorene	*N,N*-Dimethylnitrosamine (DMN) *N,N*-Diethylnitrosamine (DEN) NNK 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) Nornitrosonicotine (NNN) 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	Benzene Styrene Acrylonitrile Vinyl carbamate Vinyl chloride Vinyl bromide Ethyl carbamate Trichlorethylene Carbon tetrachloride Chloroform DMN DEN NNK NNAL NNN Butadiene	Aflatoxin B1 Aflatoxin G1 Sterigmatocystin 7,8-Dihydroxy-7,8-dihydrobenzo[*a*] pyrene and some other polycyclic hydrocarbons 17β-Estradiol 6-Aminochrysene Senecionine 4,4′-Methylene-*bis* (2-chloroaniline) (MOCA) tris(2,3-Dibromopropyl) phosphate

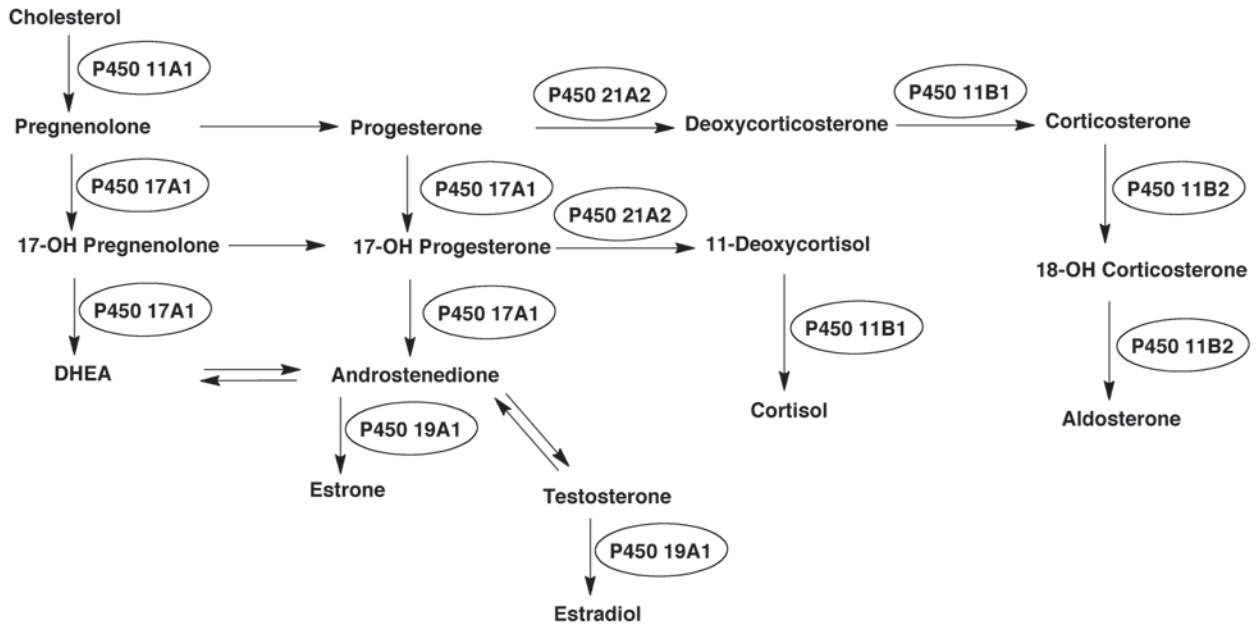

Fig. 9.12 A view of the metabolic pathway of steroidogenesis and the major P450s involved [47]. (With kind permission from Springer Science + Business Media: [149], Fig. 10.13)

aromatase reaction (conversion of androgens to estrogens) [143]. Incidentally, one of the first (1952) prominent uses of (microbial) P450s was in the practical synthesis of cortisone by the Upjohn Company [144].

The interest in P450 metabolism of steroids has been driven by several factors. One is that many steroids are used as drugs, and this section of the chapter is not independent of the one on drug metabolism. The other driving feature is inborn errors of metabolism involving steroids (Table 9.3). The subject of P450s in steroidogenesis and clinical features is treated in more depth in another chapter in this book [145] and will not be reiterated here. However, the point is made that genetic deficiencies in the steroid-metabolizing P450s usually result in obvious clinical phenotypes, as opposed to the polymorphisms in the P450s that metabolize xenobiotics.

One example of a genetic problem is P450 21A2, where about 1 in 15,000 births is affected [145]. More than 100 different gene variants have been identified in individuals presenting at the clinic. The consequences can range considerably. With P450 17A1, ~50 different genetic variants have been identified. P450 19A1 insufficiency, somewhat surprisingly, is fairly infrequent.

Some general points should be made here. Although androgens and estrogens are often considered male and female steroids, respectively, this is not really true. Both genders have some of each, and imbalances cause problems in both genders. Another point is that steroids are not restricted only to a few organs. Neurosteroids are produced by P450s in the brain and other nervous tissues. Placental steroid metabolism is important to both the mother and child [145].

Finally, some of the steroid-metabolizing P450s are drug targets themselves, in that production of androgens and estrogens is a driving factor in some tumors. Individual P450s will be discussed below, but suffice it to say for now that inhibition of estrogen production by P450 19A1 is an important aspect of many chemotherapies for breast and endometrial cancers [146], and abiraterone, an inhibitor of P450 17A1, is used in treatment of androgen-stimulated prostate cancers [147].

9.5 Approaches to Defining Catalytic Specificity of Human P450s

Knowledge of the roles of individual P450s in specific reactions (Fig. 9.8) is critical in the application of P450 biochemistry to practical issues in drug metabolism. Originally some of the P450s were purified on the basis of their catalytic activities towards certain specific drugs [14–16, 21], but even with such a strategy there are the issues of the extent of contribution of that form and the involvement of that P450 in other reactions, particularly with new substrates. Identification of the individual P450s contributing to the metabolism of a new drug candidate is routinely done in the pharmaceutical industry. This information is often requested by the US Food and Drug Administration at the time of an IND ("Investigational New Drug") application. Identifying P450s involved in oxidations is important in predicting drug–drug interactions and the extent of variation in bioavailability. In general, it is desirable to develop drugs for which several P450s have a contribution to metabolism. Drug candidates that are metabolized exclusively by a highly variant P450 (e.g., 2D6, 2C19) are often dropped from further development.

A combination of methods involving the use of human tissues and recombinant human P450s is usually used to identify P450s involved in a particular reaction, using an approach outlined earlier [30, 148, 149]. A combination of the following methods is usually done, not necessarily in a particular order. Lu has also reviewed these approaches [150].

9.5.1 Inhibitors

The reaction is demonstrated in NADPH-fortified human liver microsomes (if the reaction of interest is restricted to another tissue, then this tissue would be used instead). The effects of selective inhibitors on the reaction are examined. A list of some of the inhibitors that have been used was presented previously and a revised one is included elsewhere in this monograph by Correia and Hollenberg [85, 151].

The choice of substrate concentration is important in this and some other approaches. Ideally the effect of the substrate concentration on the rate of catalytic activity should be determined in the absence of inhibitor to determine V_{max} and K_m parameters. If this information is available, the inhibition experiments are best done with a concentration of substrate at or below the K_m, in order to observe the effect of the inhibitor on the ratio V_{max}/K_m, which is the parameter usually most relevant to human drug metabolism. If the V_{max} and K_m information is not available, an alternative is to select a substrate concentration near that expected for the in vivo plasma concentration ($C_{p,max}$ or less). Modern mass spectrometry methods have been very useful in pushing the sensitivity limits.

With regard to inhibitor concentration, ideally a range of concentrations would be used. However, if a single concentration of the diagnostic inhibitor is used, it must be selected on the basis of previous literature because nonselective effects are often observed. For instance, α-naphthoflavone (α-NF) can inhibit P450s other than P450 1A2 at high concentrations [152], and azoles inhibit many P450s at higher concentrations [85]. Use of a titration approach (concentration dependence) has merit [150].

Another general issue is the selection of a protein concentration. Microsomal proteins can bind drugs in a nonselective manner and effectively lower the free concentration of substrate or inhibitor [153, 154], which can influence the interpretation of results. Another point is that the concentration of the P450 of interest should be less than that of the drug and the inhibitor, in order for the basic assumptions about steady-state kinetics to apply (and for the reaction to remain linear during the incubation time, although some of the inhibitors are mechanism based and the loss of activity will be time dependent, requiring preincubation). A corollary of these latter points, which also apply to the other approaches that follow, is that having a very sensitive assay method is very desirable. Thus, methods such as high-performance liquid chromatography–mass spectrometry (HPLC–MS) have gained popularity.

Finally, the choice of an organic solvent (to deliver the substrate) is an issue. Most P450 substrates are hydrophobic. Ideally the substrate should be dissolved in H_2O or very little organic solvent, but this may not be possible with many drugs. Several examinations of the effects of individual solvents on human P450s have been published [155, 156]. Some very hydrophobic substrates (e.g., cholesterol) should be delivered in cyclodextrins [157].

In principle, the extent of inhibition of a reaction by a P450-selective inhibitor indicates the fraction of that reaction attributable to that P450. For instance, if a 1 μM concentration of quinidine (a P450 2D6 inhibitor) inhibits 50% of a reaction, then 50% of that reaction may be attributed to P450 2D6. To obtain a more global view than possible with a single liver sample, a pooled set of microsomes (e.g., from ≥10 samples, balanced on the basis of liver weight or protein) is generally used for the inhibition assays. However, if one desires to examine the differences among individuals in terms of the contribution of a P450, then doing several experiments with individual liver samples is the approach to use.

9.5.2 Correlations

Another approach with a set of human tissue microsomal samples is to measure the new reaction of interest in each and attempt correlation with rates of marker activities (for individual P450s) [25]. Lists are also published in this monograph by Correia and Hollenberg [85] and elsewhere [158, 159].

Correlation can be done by plotting the specific activity for the new reaction versus the marker reaction (Fig. 9.11). In principle, the correlation coefficient r^2 estimates the fraction of the variance attributable to the relationship between the two activities, i.e., the fraction of the activity catalyzed by the particular enzyme (assuming that all of the marker activity is catalyzed by this enzyme). In some cases, excellent correlations have been reported [26, 60]. An alternative method of analysis is a Spearman rank plot, which has some deficiencies but avoids the overweighting of unusually high or low values [27].

Although the approach works well when high correlation coefficients are generated, the method

is less useful when several P450s contribute to a reaction, i.e., $r^2 < 0.4$. The results should, in all cases, be considered in the context of results obtained with other approaches.

9.5.3 Antibody Inhibition

The points raised in the above section, Inhibitors, apply to antibodies as well. Antibodies are used to inhibit activities in human liver (or other tissue) microsomes and are of several general types: (1) polyclonal antibodies raised against purified animal P450s, (2) polyclonal antibodies raised against purified human P450s, (3) monoclonal antibodies raised against purified human P450s, (4) polyclonal antibodies raised against peptide fragments of P450s, and (5) antibody phage display library antibodies selected for recognition of individual P450s.

At this time, almost all antibodies raised against intact P450s have been generated using recombinant P450s (or against peptides), in contrast to early work in the field with P450s isolated from liver and other tissues. Another point to make is that not all antibodies inhibit catalytic activity. Further, specificity in one immunochemical assay (e.g., electrophoretic/immunoblotting) does not necessarily implicate specificity in another (immunoinhibition).

Three points should be made in designing immunoinhibition experiments. (1) The concentration of antibody should be varied and increased to the point where the extent of inhibition is constant. (2) A nonimmune antibody should be used as a control, using the same concentrations as with the antibody raised against the P450. (3) The antibody should be shown not to inhibit reactions known to be attributable to other P450s. Immunoglobulin G fractions are generally preferred in that they produce less nonspecific inhibition than crude preparations such as sera. Polyclonal antibodies can vary in their specificity and titer from one animal to another and from one bleed to another, so constant properties cannot necessarily be assumed. In principle, monoclonal antibodies and antibodies eluted from phage display libraries should not vary (among individual preparations), although this has not always been the case with monoclonals.

In general, antibodies are often selective for individual P450 families/subfamilies, e.g., 1 versus 2A versus 2B versus 2C, etc., but cross-reaction among families can be detected, and in some cases the (P450) sites of cross-reactivity have been identified [160]. Achieving selectivity among individual P450 subfamily members (e.g., P450 3A4 versus 3A5 versus 3A7) is more difficult. With polyclonal antibodies, this can be achieved by cross-absorption [161]; with monoclonals and phage display libraries, this can be done by selection. The point should be made that any selectivity demonstrated among classes of animal P450s (e.g., rat P450 families) cannot be assumed to carry over to human P450s.

Anti-peptide antibodies have become popular in recent years and have two major advantages: (1) peptides can be synthesized and readily purified by HPLC, avoiding the need to express and rigorously purify P450 proteins (although demonstration of purity by HPLC, capillary electrophoresis, and mass spectrometry is still in order), and (2) peptides can be selected for use as antigens by sequence comparisons, favoring specific regions.

Phage display antibody libraries are relatively new and have been used in a few P450 applications to date [162]. These have a number of advantages, including potential selectivity due to the large number of potential antibodies in libraries, the ability to avoid animal protocols, the immediate availability of libraries (as opposed to waiting on animals to develop antibodies), the consistency of reproduction of the proteins propagated in bacterial systems, and the ability to include a second “epitope tag” for recovery.

9.5.4 Demonstration of Reaction with Recombinant P450

In early work in this field, this point would have been the demonstration of the reaction of interest with an enzyme purified from tissue. Today P450 proteins are generally produced in recombinant systems and seldom purified from tissue

sources. In routine practice in the pharmaceutical industry, new reactions are examined with a battery of the major recombinant human (liver) P450s, many of which are available from commercial sources. Systems used for expression include bacteria, yeast, baculovirus (-infected insect cells), and mammalian cells. The P450 need not be purified for these comparisons but must have suitable provision for NADPH-P450 reductase in a crude system (and cytochrome b_5 in certain cases).

Usually activity results obtained with several of the major P450s are compared to each other and to those obtained with tissue microsomes, in order to put the work in context. Ideally assays are done at several substrate concentrations and the parameters k_{cat} (V_{max}) and k_{cat}/K_m are obtained. These values should be normalized on the basis of P450 concentration, in that any values based on mg protein for the expression system cannot be used for comparisons with tissue microsomes. In principle, the k_{cat} (total P450 basis) should be at least as high for the recombinant reaction than the tissue microsomes. A more realistic way to make a comparison is to immunoquantify the amount of the particular P450 in the tissue microsomes and then use this value in correcting the microsomal k_{cat} for comparison to the recombinant system. The matter of scaling these parameters to generate predicted microsomal (or in vivo) rates from in vitro experiments with recombinant enzymes is not trivial, but there has been considerable progress in this area and there is commercial software in wide use [163].

9.6 Interindividual Variation

9.6.1 Genetic

Variability in patterns of drug metabolism has been recognized for some time, even before the discovery of P450s. For instance, the field of pharmacogenetics had been identified by the 1950s [44, 164] and the early work of Remmer [2] showed the influence of barbiturates upon drug metabolism. Further, a number of congenital defects in steroid metabolism were known and some could be attributed to alterations in specific hydroxylations [165]. Much of the subsequent work on inducibility has been done in experimental animal models [1] and, later, in cell culture.

In the 1960s and 1970s, a number of accounts appeared describing variations in rates of metabolism of drugs in human liver biopsy samples [28]. The first characterization of a monogenic variability in a human drug-metabolizing P450 was the work of Smith with debrisoquine [11], as well as Tucker and Lennard [12], which was paralleled by the work of Dengler and Eichelbaum on sparteine [13]. This polymorphism was first described in the context of EMs and PMs (Fig. 9.4) [62, 63]. These polymorphisms were first studied at the level of the phenotype, i.e., pharmacokinetics and in some cases unusual responses to drugs due to reduced metabolism [166]. The area of pharmacogenetics (now expanded to "pharmacogenomics") was facilitated by the identification of the P450 enzymes involved in the drug metabolism phenotypes and particularly by the development of molecular biology, which allows the precise characterization of genetic differences between individuals. The majority of the allelic differences are SNVs, or single base changes. As anticipated from previous knowledge of pharmacoethnicity, many of these SNVs and polymorphisms show racial linkage. (Again, a polymorphism is generally defined as a $\geq 1\%$ frequency of an allelic variant in a population; below this frequency, the term "rare allele" is applied or, in the case of a very detrimental allele, a mutant or "inborn error of metabolism." Therefore, as mentioned earlier, the terms "variant" and "SNV" will be used to include both, not distinguishing for frequency.)

The debrisoquine polymorphism is now well understood in terms of P450 2D6 and has been a prototype for research in this area. The characterization of the gene [34] yielded a basic understanding of the PM phenotype. The incidence of the PM phenotype is $\sim 7\%$ in most northern European populations, with different phenotypic incidence (and SNVs) in other racial groups [62, 67, 167, 168]. More than 160 allelic variants are now known, and 98% of the PMs in northern European populations can be accounted for by four

variant alleles [67, 169]. A nomenclature system has been set up for P450 alleles (using the suffixes *1 (where *1 is the "wild type," or most common gene), *2, *3…) and is maintained at http://www.cypalleles.ki.se. Reference to this database will be made with most of the individual P450s.

Several P450 2D6 allelic variants clearly lead to the PM phenotype, for a variety of reasons. A relatively rare case is a gene deletion (*5) [170]. The most common (Caucasian) PM phenotype is an SNV that leads to aberrant RNA splicing (i.e., in splice site) and no mRNA or protein (*4). Other alleles involve deletions (e.g., *5), frameshifts (e.g., *3A), and coding for proteins with either intrinsically low catalytic activity or instability (reduced half-life). These general patterns have been seen in other P450s (and other genes). In addition to the EM and PM phenotypes, there is also a "very extensive metabolizer" (or "ultrametabolizer," UM) phenotype (Fig. 9.4), due to gene duplication (*2XN). A Swedish family was identified with 13 gene copies, in principle leading to 13 times more enzyme [63]. The level of hepatic P450 2D6 and a parameter of in vivo debrisoquine metabolism (the urinary metabolic ratio = urinary debrisoquine/4-hydroxydebrisoquine) vary ~10^4-fold among people (Fig. 9.4). With P450 2D6 and several other P450s, the alleles describing the most commonly observed high and low levels of metabolism have been described, but the kinetic parameters for many of the alleles have not been determined by heterologous expression and measurements. This is still the general case with most of the human P450s. P450 2D6 is regulated by a hepatic nuclear factor (HNF) element [171] but is not considered to be inducible by xenobiotics. With many other P450s, there is regulation and variability due to noncoding region SNVs, levels of inducers consumed, and interactions between P450s and transporters such as P-glycoprotein [66, 172] may influence the phenotype.

Although the level of P450 2D6 may have a dramatic effect on the metabolism of certain drugs (Fig. 9.4), no other striking biological changes have been reported in PMs (but see some of the epidemiology under Sect. 7.12.7). This appears to be the general case for many of the hepatic P450s primarily involved in the metabolism of xenobiotics, and few observable physiological effects have been reported in transgenic mice in which these genes have been deleted [128]. As pointed out earlier, however, deficiencies in some of the steroid-hydroxylating P450s can be very debilitating or lethal [145, 165]. In general, the variation in the levels of these "more critical" P450s is limited in most of the population, compared to the xenobiotic-metabolizing P450s in which an order of magnitude variation is not unusual [52].

Another general point to make is that, in contrast to some animal models [173], human P450 expression shows little if any gender differences [64].

9.6.2 Environmental Variation

Interindividual variability of P450 activity can be due to genetics or to environmental factors, i.e., anything that is not genetic. These factors also give rise to intraindividual variations, which can be equally important in predicting how an individual will respond to a drug. These variations may be caused by drugs, food, tobacco, alcohol, and other influences. The three major issues here are enzyme induction, downregulation, and inhibition. These topics are dealt with elsewhere in the book and will only be discussed briefly, insofar as they relate to human P450s. One other topic, enzyme stimulation, is also discussed below.

When developmental differences are seen in humans, they tend to be relatively soon after birth (e.g., P450 3A4, 3A7 [174, 175]), and changes in expression in the elderly have not been very dramatic [176–178].

9.6.2.1 Induction

Induction is a process that is relatively common among the P450s involved in the oxidation of xenobiotic chemicals (second column of Table 9.1). The overall process can be seen as an adaptive one, at least in some cases, in which a person responds to a chemical in the environment by synthesizing an enzyme to metabolize that compound or a set of similar ones.

The general model is one of transcriptional regulation, based on a paradigm developed for the steroid nuclear receptor family (Fig. 9.13), which is considered in more detail in Chap. 10. A ligand is bound to a cytosolic receptor, which facilitates heterodimer formation with another protein. This complex then translocates to the nucleus and binds to a specific nucleotide sequence (5′) upstream of the P450 structural gene. Coactivator proteins are often recruited to the complex. This process has the net effect of chromatin remodeling and opening the promoter site to allow RNA polymerases binding and initiation of transcription.

Several major systems are known to be involved with (human) P450s. The AhR system involves the AhR and AhR nuclear transporter (ARNT) proteins, regulating P450s 1A1, 1A2, 1B1, and 2S1. The constitutive androgen receptor (CAR) dimerizes with the retinoid X receptor (RXR), which is loaded with retinoic acid. CAR can bind a strong ligand (e.g., 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCBOPOP)) but usually acts without a ligand. Recent evidence indicates that phenobarbital, the classic barbiturate inducer, binds to the epidermal growth factor receptor (EGFR), leading to a cascade of extracellular signal-regulated kinase (ERK) phosphorylation, and then dephosphorylation of CAR (at Thr-38) leads to transport of the CAR–RXR*α* complex to the nucleus and gene activation [179]. This process induces subfamily 2C and 2B P450 genes, plus possibly some others. The pregnane X receptor (PXR) binds a number of steroids, drug, and other ligands and, like CAR, heterodimerizes with retinoid-activated RXR*α,* moves to the nucleus, and activates the transcription of P450 subfamily 3A genes, particularly

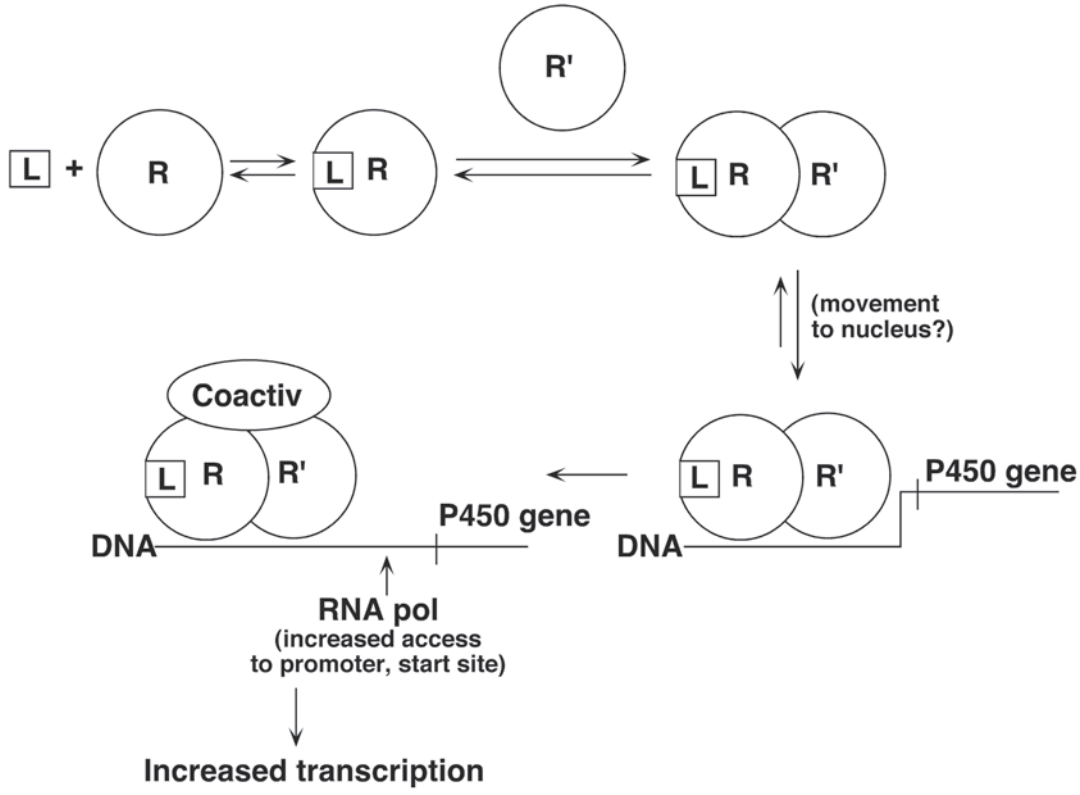

Fig. 9.13 Generalized model for regulation of P450 genes by induction. *L* ligand, *R* receptor, *R′* partner protein for heterodimer of R, *Coactiv* coactivator, *RNA pol* RNA polymerase. (With kind permission from Springer Science + Business Media: [149], Fig. 10.6)

P450 3A4. The peroxisomal proliferator-activating receptor α (PPARα) binds fatty acids and a number of hydrophobic drugs, heterodimerizes with retinoid-activated RXRα, and induces P450 4A11 and 4X1 [180].

Some of the steroid-oxidizing P450s are regulated by adrenocorticotropic hormone (ACTH) and cyclic adenosine monophosphate (AMP) pathways [181].

Evidence has been presented that some P450s are regulated at post-transcriptional levels, including stabilization of mRNA or protein [182]. The regulation of P450 2E1 is extremely complex, at least in animal models [183, 184]. Several reports of epigenetic regulation of P450s have appeared, including gene methylation (e.g., P450s 2A13, 2E1, 2R1, 5A1, 8A1, 19A1, 24A1, 27A1, 27B1, 2W1 [185, 186]), microRNAs (e.g., P450s 1B1, 2E1, 3A4, 24A1) [187], and histone acetylation (e.g., P450s 2A13, 2E1, 46A1 [188]).

9.6.2.2 Downregulation of P450s

It should be pointed out that several of the P450s can be downregulated by cytokines, and the result has practical significance in the impairment of drug metabolism in individuals with colds or flu or who have received vaccinations [189].

Another phenomenon observed in rat models is the downregulation of some constitutive P450s by the same chemicals that induce others, e.g., phenobarbital and 3-methylcholanthrene [190]. The mechanism of this response is at the transcriptional level [191] but beyond this mechanism remains unknown. Whether this phenomenon is operative in humans (in vivo) is also unknown.

9.6.2.3 Inhibition

The subject of P450 inhibition is also treated separately in this book (Chap. 5) [85], and this section is brief, focused on human P450s. A relatively extensive set of P450 inhibitors is now available, and many of these can be used in a diagnostic way for "reaction phenotyping," i.e., identifying which P450s catalyze a newly discovered reaction in tissue microsomes (see Sect. 5).

Inhibition of human P450s is an important practical matter on drug discovery/development and in clinical practice (Table 9.6) (medicine.iupui.edu/clipharm/ddis). P450 inhibition has the same effect as a genetic deficiency (attenuation of drug metabolism, leading to enhanced pharmacological response), but can be even more problematic because of temporal changes. For instance, some drugs can produce a delayed response for various reasons, and the pharmacokinetics of a drug (substrate) may vary with time. Another important point is that not all human P450 inhibitors are drugs. For instance, an inhibitor in grapefruit (bergamottin) explains the interaction with P450 3A4 [88]. A number of herbal medicines contain P450 inhibitors that attenuate drug metabolism [192].

9.6.2.3.1 Reversible Inhibitors

Competitive inhibitors are common. They act by binding in the active site, in competition with the substrate. For instance, two substrates of P450 2D6 would be expected to compete for access to the area surrounding the iron atom. This behavior is described by the simple equation

$$v = V_{\max} \cdot \frac{[S]}{K_{\mathrm{m}}\left(1+\frac{[I]}{K_{\mathrm{I}}}\right)+[S]}.$$

Noncompetitive inhibition is the result of an enzyme interaction of a ligand at a site other than the substrate-binding site. The equation

$$\frac{1}{v} = \frac{1}{V_{\max}}\left[1+\frac{[I]}{K_{\mathrm{I}}}\right]+\frac{K_{\mathrm{m}}}{V_{\max}}\left(1+\frac{[I]}{K_{\mathrm{I}}}\right)\frac{1}{[S]}$$

indicates that the K_{m} will not change but the $V_{\max}$ (k_{cat}) will.

In *uncompetitive inhibition,* the inhibitor combines with only the ES form of the enzyme, and the inhibitor constant K_{I} is based on the interaction of the inhibitor with this complex,

so that

$$\frac{1}{v} = \frac{1}{V_{\max}}\left(1+\frac{[I]}{K_{\mathrm{I}}}\right)+\frac{K_{\mathrm{m}}}{V_{\max}}\cdot\frac{1}{[S]},$$

and in a classic Lineweaver–Burk double reciprocal plot (1/*v* versus 1/[S]), two parallel lines are obtained, i.e., both V_{max} and K_I change [193].

In practice, the most common type of reversible inhibition relevant to human P450s and drug metabolism is the competitive mechanism. Uncompetitive inhibition is very rare; one (non-P450) example is the inhibition of steroid 5*α*-reductase by the drug finasteride [194]. An example of noncompetitive inhibition is that of cholesterol blocking the oxidation of nifedipine and quinidine by P450 3A4, even though cholesterol is also a substrate for the enzyme [157].

9. 6. 2. 3. 2 Irreversible Inhibition

For several reasons these mechanisms are commonly seen in P450 reactions. In a sense, they are more problematic than competitive inhibitors, in that the inhibition is more persistent, i.e., the enzyme is generally inactivated and activity will not be restored until new synthesis is completed.

Metabolite Intermediates

Metabolite intermediate complexes are formed by the oxidation of amines to C-nitroso compounds or from oxidation of methylene dioxyphenyl compounds to carbenes [195]. These bind extremely tightly to ferrous P450 iron. Both of the bound forms are characterized by their 455-nm absorption bands, which can be produced in in vitro experiments. A classic example is seen with troleandomycin (TAO) and P450 3A4 [196]. These complexes can be disrupted by $K_3Fe(CN)_6$ oxidation of the iron (in vitro).

Covalent Binding

Covalent binding, where *σ* chemical bonds are formed, is the result of the generation of electrophilic species in the course of P450 oxidation of compounds. The binding may occur to the heme, the apoprotein, or both (i.e., cross-linking, a rare but documented event [197, 198]). A number of chemical moieties are notorious for such mechanism-based inactivation, including acetylenes, some terminal olefins, and cyclopropylamines [199]. The destruction of heme is probably due to very transient species that are generated during catalysis and do (usually) not leave the enzyme. In many cases, the covalent binding to protein is restricted to the P450 that activates the compound, which is one of the marks of an enzyme intermediate. However, in some cases there may be reactions with the P450 and with other proteins. In this case, the reactive products are long-lived and there is concern not only about the (P450) enzyme inhibition but also potential toxicity due to modification of other proteins.

Along with a chapter on P450 inhibition (Chap. 5) [85], inhibitors of each human P450 are discussed in the appropriate Sects. (7.X.6) of this chapter (X indicates each of the 57 P450s).

9.6.2.4 Stimulation

Enzyme stimulation is an increase in enzyme activity resulting directly from the addition of a chemical. This is a somewhat unusual phenomenon in enzymology, usually relegated to classically allosteric systems [200]. The concept is that a chemical stimulates the catalytic activity of an enzyme. This cooperativity may be considered in two aspects. One is *homotropic* cooperativity, in which a chemical stimulates its own biotransformation. This is usually manifested in sigmoidal (S-shaped) plots of *v* versus *S*. *Heterotropic* cooperativity is the stimulation of catalytic activity by direct addition of a different compound.

Both of these phenomena have been observed with P450s in vitro. Heterotropic stimulation was reported with animal-derived P450 systems [201, 202] and then human systems [203]. Homotropic cooperativity was reported later, actually first with human systems [204, 205]. Homotropic cooperativity can be shown in hepatocytes [206], but it may be unrealistic to observe this phenomenon in vivo. Evidence for in vivo cooperativity comes from a number of studies with experimental animals [202, 207]. Whether this phenomenon presents itself clinically is unknown. It would not generally be desirable in that the effects on pharmacokinetics would be rather unpredictable.

At least four pieces of evidence suggest that such behavior is possible: (1) homotropic cooperativity has been reported in hepatocyte cultures [206]; (2) an early experiment with neonatal mice (individual P450s unknown) by Conney's group indicated the immediate enhancement of

an activity by flavones [202]; (3) the work of Slattery and Nelson with rats shows interaction between caffeine and acetaminophen that imply such behavior [208]; and (4) quinidine enhanced the in vivo oxidation of diclofenac in monkeys, in a manner consistent with in vitro human work [207, 209]. The first example (hepatocytes) relates to homotropic cooperativity, but this would be very hard to demonstrate in vivo, except perhaps in the interpretation of unusual nonlinear pharmacokinetics, if induction can be ruled out. The other three (in vivo) are cases of heterotropic cooperativity. If stimulation does occur in vivo, it is a phenomenon that has been very difficult to predict (even in vitro), and in the case of P450 3A4 substrates, the situation would probably be further complicated by issues involving P-glycoprotein behavior (and P-glycoprotein also shows cooperativity of its own [210]).

The mechanistic basis of P450 stimulation has been studied extensively. Some aspects of P450 stimulation will be treated under the topic of P450 3A4 (Sect. 7.20.4), with which much of the work has been done. An open question is whether such behavior occurs in humans. Many classic allosteric enzymes have distinct regulatory sites for binding chemicals, but to date there has been no clear evidence for this. One of the early proposals was that the second ligand fits into the canonical active site, near the substrate [205]. This view was advanced in a number of indirect studies that supported the concept [211, 212]. Although a number of different (human) P450s have exhibited cooperative behavior, much of the emphasis has been on P450 3A4. This was the first human P450 to show heterotropic cooperativity [204, 205]. In addition, its broad substrate specificity allows the examination of more chemicals, both substrates and effector molecules. Although there had been many postulates of multiple ligand occupancy in P450 3A4, this was first demonstrated with X-ray diffraction, i.e., two ketoconazole molecules in P450 3A4 [213].

The physical presence of two ligands in an active site can be readily linked to sigmoidal kinetics if activity towards the substrate is dependent upon the presence of two substrates [214].

Two X-ray structures of P450 3A4 have reported a single steroid molecule bound at a peripheral site [215, 216], although the relationship to function is unclear. Evidence from this laboratory [217, 218] and others [219] has provided evidence that binding of at least some substrates to P450 3A4 involves rapid binding to a peripheral site followed by a slower movement to the heme area. Evidence for a similar course of substrate movement has been observed with P450s 1A2 and 19A1 [214, 220].

Two-ligand occupancy of a P450 active site, with the ligands stacked together, has now been observed with bacterial P450s 107 [221] and 158A2 [222] and human P450s 2C8 [223], 3A4 [213], and 21A2 [42]. The case that cooperativity is due to multiple-ligand occupancy now has physical support, but the question arises as to why cooperativity has not been seen in P450s that do have two ligands, e.g., P450 2C8 [223]. (With P450 21A2, there was no evidence for cooperativity but there was for two affinities [42].)

9.7 Individual Human P450 Enzymes

Each of the 57 human P450 genes/gene products will be covered here. Clearly much more information is available about some than others. Points to be covered with each, when possible, include sites of expression and relative abundance, regulation, genetic variation, substrates and reactions, structure, inhibitors, and clinical issues. It must be emphasized that this chapter is not intended to be comprehensive, and the literature accumulates rapidly; the reader is encouraged to do further literature searches for each P450 of interest.

9.7.1 P450 1A1

9.7.1.1 Sites of Expression

The gene has seven exons, and the cDNA region is ~70% identical to that of the closest relative, P450 1A2. P450 1A1 is expressed in fetal liver but not at appreciable levels in adult liver [224–226]. P450 1A1 can be induced in primary human he-

patocyte cultures [227]. The dominance of hepatic P450 1A2 over 1A1 in vivo may be due to preferential induction of P450 1A2 > 1A1 at low doses of inducers (a phenomenon established in rats [228]) or to the presence of factors in liver that are not preserved in hepatocyte cultures.

P450 1A1 is expressed in human lung and was partially purified [18]. One estimate of a median level of P450 in human lung [229] was 6.0 pmol P450 1A1 in nonsmokers' lungs ($n = 7$), 16 pmol/mg in smokers ($n = 18$), and 19 pmol lung protein in ex-smokers ($n = 7$). The variation in levels of P450 1A1 is very high (> 100-fold) [18, 229], as suggested from earlier work in which only benzo[*a*]pyrene hydroxylation was used as an indicator [230].

P450 1A1 is also expressed in placenta [231] and peripheral blood cells (lymphocytes, monocytes) [232], and these tissues have been used in many studies. Expression (at least at the mRNA level) has been reported in a number of other extrahepatic tissues, including pancreas, thymus, prostate, small intestine, colon, uterus, and mammary gland [233].

Another aspect of P450 1A1 expression involves mitochondria. P450 1A1 has both endoplasmic reticulum and mitochondrial-targeting domains [234] and distributes into both organelles, utilizing adrenodoxin for functional electron transfer in the mitochondria.

9.7.1.2 Regulation

The induction of P450 1A1 has been studied extensively and has been discussed elsewhere in this series [235]. Briefly, the AhR resides in the cytosol and, when activated by binding of an appropriate agonist, loses the accessory protein Hsp90 and dimerizes with the ARNT protein, moving to the nucleus and interacting with a xenobiotic-responsive element (XRE) to initiate transcription (Fig. 9.13, with R = Ah receptor, R^1 = ARNT, and L = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or other inducer). A number of details regarding this scheme remain to be elucidated, e.g., roles of coactivators, whether an endogenous ligand exists, and if so what it is. The list of inducers reported from in vitro studies includes TCDD and is quite long. The list of compounds for which in vivo evidence of induction is more limited but is generally accepted includes cigarette smoke, heterocyclic amines, polychlorinated biphenyls [236], and some drugs (e.g., omeprazole [237]).

At least six human AhR genetic variants have been identified and found to vary in functional activity but surprisingly (based on mouse work) only ~two-fold [238].

In Michigan Cancer Foundation-7 (MCF-7) breast cancer cells, regulation of P450 1A1 (via AhR) is dependent on the Ca^{2+}/calmodulin/CaMKIα pathway [239]. Epidermal growth factor (EGF) has been reported to downregulate AhR in human keratinocytes [240, 241]. There is also cross talk of AhR systems with the estrogen receptor (ER)α [240]. CAR transcriptionally activates both P450 1A1 and 1A2 genes through a common 5′-flanking region regulatory element [242]. Liver X receptor α (LXRα) also regulates human P450 1A1 [243]. In (human) HepG2 cells, P450 1A1 gene regulation by ultraviolet (UV) light (UVB) involves cross talk between AhR and the nuclear factor NFκB [244], which also has relevance to an inflammatory response [245] and possibly humans.

An unusual mechanism of regulation involves inhibition of the clearance of an endogenous AhR agonist, 6-formylindolo[3,2-*b*]carbazole (FICZ; a tryptophan photodegradation product), as a mechanism for activating AhR [246]. Another unusual regulatory mechanism, demonstrated only in mice thus far, involves activation of AhR by modified low-density lipoprotein (LDL) [247]. Finally, 1-nitropyrene has been reported to stabilize mouse P450 1A1 mRNA via an Akt pathway [248].

9.7.1.3 Genetic Variation

Currently at least 13 alleles are known, plus another seven single nucleotide polymorphisms (SNPs) in which the haplotype has not been determined (http://www.cypalleles.ki.se).

As mentioned earlier, there is also information available about genetic variation in the AhR, which controls P450 1A1 transcription [238].

Polymorphism in the inducibility of benzo[*a*]-pyrene hydroxylation activity has attracted considerable interest following the early reports of

Shaw and Kellerman [108, 109] that the induction in lymphocytes of smokers can be associated with susceptibility to lung cancer. The link to lung cancer has been studied extensively, but few general conclusions can be reached. Smoking clearly induces levels of lung P450 1A1 [111, 229, 249]. Some epidemiological investigations have linked the *2A (*Msp*I) and *2B (I462V) polymorphisms to lung cancer incidence in Japanese [116], but this association has not been reproduced in other studies with Caucasians [117, 118]. These two alleles are in linkage disequilibrium [119]. Two studies with recombinant human P450 1A1 have not shown a major difference in any catalytic activities due to the substitution at codon 462 [120, 121]. Although there is a general consensus that phenotypic variation in the inducibility of P450 1A1 is observed, extensive searches have not associated the inducibility with any known polymorphisms in the P450 1A1, AhR, or ARNT genes [250, 251].

9.7.1.4 Substrates and Reactions

This enzyme was first explored in the context of an aryl hydrocarbon hydroxylase, using fluorescence assays that measure primarily the 3-hydroxylation of benzo[*a*]pyrene [107]. (It should be noted that the fluorescence assay also picks up other fluorescent products, e.g., 9-hydroxybenzo[*a*]pyrene, and that other P450s also catalyze the 3-hydroxylation reaction, e.g., P450 2C9 in human liver [252].) Another classic model reaction used for P450 1A1 is 7-ethoxyresorufin *O*-deethylation [253, 254], but a number of other P450s also catalyze this reaction. Human P450 1A1 oxidizes benzo[*a*]pyrene to a variety of products [255, 256]. Many other polycyclic hydrocarbons are substrates for P450 1A1 and have been studied extensively [257, 258] (Fig. 9.10). Some heterocyclic and aromatic amines can also be activated by P450 1A1 (Fig. 9.10) [259]. P450 1A1 does not appear to play a major (in vivo) role in the metabolism of many drugs, possibly because of its locations of expression.

Human P450 1A1 activates aminomethylphenylnorharman, a fusion product of norharman [260], but not as well as P450 1A2. P450 1A1 is involved in the detoxication of the important toxic natural product aristolochic acid [261]. The EGFR antagonist erlotinib is activated to a reactive electrophile by human P450 1A1 [262]. Some substituted benzothiazole compounds can be activated (quinones, *N*-hydroxylation) by human P450 1A1 [263].

TCDD and other dioxins can be oxidized (albeit slowly) by P450 1A1 enzymes, but rat P450 1A1 is more active than human P450 [264]. 1-Nitropyrene is deactivated by P450 1A1, to a product that does not induce the tumor suppressor p53 [265].

Cytochrome b_5 has generally been considered not to stimulate P450 1A1 [266], but some examples have been published [267].

9.7.1.5 Structure

Early work on pharmacophore models for rat P450 1A1 was done by Jerina's group [268]. Some homology modeling was done by Lewis [269]. The lack of effect of interchanging Val and Leu at position 462 has already been mentioned [120, 121].

An X-ray crystal structure of human P450 1A1 has been published by Scott and her associates [270]. Because the structure contains α-NF, it can be compared directly with the structures of the related proteins P450 1A2 [271] and P450 1B1 [272]. The planar region of α-NF is packed flat against the I-helix, with the 2-phenyl substituent oriented towards the iron atom of the heme. π–π stacking with Phe-224 was observed [270].

As in the case of P450 1A2 (Sect. 7.2.5, *vide infra*), α-NF has the site of oxidation (5,6-epoxidation [256]) furthest away from the iron atom and the observed P450 1A1 α-NF structure is presumably not a catalytically productive complex. Docking studies could place α-NF in a juxtaposition to explain the oxidation [270] (which is known to be slow, but faster with P450 1A1 than P450 1A2 [256]).

A combinatorial approach has been used to "mix" human P450 1A1 and 1A2 to define residues that contribute to the "identity" of each of these two P450s [273].

9.7.1.6 Inhibitors

Despite the long interest in this enzyme, the list of inhibitors is relatively short, and many inhibitors show overlap with P450s 1A2 and 1B1 [274]. For instance, α-NF is often used as inhibitor but is more effective against P450 1A2 [274, 275]. Another inhibitor is ellipticine [159]. 1-(1′-Propynyl)pyrene and 2-(1′-propynyl)phenanthrene were found to be selective P450 1A1 inhibitors when compared against human P450s 1A2 and 1B1 [274].

More efforts have been made to synthesize new inhibitors of P450 1A1 [276]. Several organoselenium compounds are inhibitors [277], as well as flavonoid derivatives [278]. The natural product rhapontigenin is a low K_I inhibitor of P450 1A1 [279]. The furanocoumarin chalepensin is a mechanism-based inactivator of P450 1A1 [280]. Finally, the endogenous (tryptophan photolysis product) AhR ligand FICZ (*vide supra*) is a high-affinity ligand/inhibitor of human P450 1A1 [246].

9.7.1.7 Clinical Issues

Because of a rather limited role of P450 1A1 in drug metabolism, there are no real pharmacokinetic issues. The issue with P450 1A1 is induction and a possible role in chemical carcinogenesis. Work with animal models shows that P450 1A1 inducers can be cocarcinogens [70, 103]. Thus, regulatory agencies have tended to look unfavorably at induction of P450 1A1 by potential drugs in animal models. However, the point should be made that there is presently little experimental or epidemiological evidence to support this hypothesis, and Ah inducers can afford protection from cancer in some animal models [103] (Figs. 9.9 and 9.10).

Very little evidence has been obtained that the common genetic variations in human P450 1A1 have functional consequences with carcinogen metabolism, e.g., Ile-462 versus Val-462 [120]. However, genetic variations have been examined for relationship to overall cancer [281] and to breast [282], colorectal [283], lung [284], oral [285], and endometrial [286] cancers. The overall evidence for relationship in any case is still very limited.

9.7.2 P450 1A2

9.7.2.1 Sites of Expression

As mentioned earlier, human P450s 1A1 and 1A2 both have seven exons and 70% sequence identity in their coding regions. These two genes both show similar patterns of regulation by the AhR system, but P450 1A2 is essentially only expressed in the liver [233], probably due to the involvement of HNF in its regulation (*vide infra*). Several lines of evidence indicate that the level of expression is substantial (Fig. 9.2), ~6–13% of the total P450 on the average, with levels varying ~40-fold among individuals (Figs. 9.5 and 9.11). A similar fold variation is seen in the in vivo metabolism of the marker drug caffeine [61].

One LC–MS proteomic analysis of human liver microsomes yielded a mean of 29 pmol P450 1A2/mg microsomal protein (range 2.9–104) [55] while another yielded 11–18 pmol/mg microsomal protein [54].

Occasional reports cite mRNA expression in some extrahepatic tissues, e.g., colon [287]. Extensive searches have not found expression in human lung [233].

9.7.2.2 Regulation

The variability and inducibility of P450 1A2 have been recognized for some time, indirectly, going back to studies on phenacetin metabolism by Conney and his associates [288]. The characterization of P450 1A2 ("$P450_{PA}$") as the low K_m phenacetin *O*-deethylase [14] led to some interpretation of the earlier results. P450 1A2 was shown to be the caffeine N^3-demethylase [60], and the 40-fold variation in levels of liver P450 1A2 is reflected in the 40-fold variation in some in vivo parameters of caffeine metabolism [61]. Some of Vesells's earlier work on the metabolism of antipyrine in twins suggests a role for genetic polymorphism in P450 1A2 activity [4], and a more recent twin study confirms the strong genetic component of caffeine demethylation [289].

One complication with genetic polymorphism, as with P450 1A1 (*vide supra*), is the inducibility. Because of the availability of markers of hepatic P450 1A2 function (phenacetin is no longer used, due to its carcinogenicity in animal

tests, but caffeine and theophylline are), demonstrating in vivo changes in P450 is relatively easy to do and the effects are consistently seen, at least quantitatively. The mechanism of induction appears to be similar to that of P450 1A1 (Fig. 9.13), with expression restricted to the liver because of the need for HNFα [290]. An interesting observation made recently in mice is that the inducer 3-methylcholanthrene causes a persistent induction (of P450 1A1) in liver, lasting beyond the time suggested by pharmacokinetic expectations [291]. One interpretation is that a P450 1A2-generated metabolite is involved. Further details and any relevance to humans remain to be established. With animal P450 1A2, one mechanism of induction involves protein stabilization, e.g., by isosafrole-derived products [292]. Whether or not this mechanism is relevant in humans is unknown. Reported inducers include cigarette smoking, charbroiled food (presumably polycyclic hydrocarbons and heterocyclic amines), cruciferous vegetables, vigorous exercise [293], and the drug omeprazole (actually a metabolite) [294].

The nuclear receptor LXRα has been found to be involved in the regulation of both human P450 1A2 and 1A1 [295]. Dehydroepiandrosterone (DHEA) has been reported to downregulate human P450 1A2 through an unusual mechanism, destabilizing the mRNA [296]. P450 1A2 phosphorylation has also been reported in vivo [297]

Table 9.9 Some drug substrates for human P450 1A2[a]

Drug[a]	Reference
Acetaminophen (3′)	[304]
Antipyrine (4,3-methyl)	[305]
Bufuralol (1,4)	[306]
Caffeine (3)	[60]
Clozapine	[67]
Olanzapine	[67]
Ondansetron (7,8)	[307]
Phenacetin	[14]
Tacrine	[308, 309]
Theophylline (1,3,8)	[310]

[a] Site of oxidation indicated in some cases. See also Rendic [51]

9.7.2.3 Genetic Variation

Although many early studies in this field discounted a genetic contribution to the variability of P450 1A2 levels due to lack of sharp breaks in frequency distribution plots [132, 298], the gene has been shown to be rather polymorphic/variable. At least 41 alleles are known [168], and five additional SNPs remain to be characterized for haplotype (http://www.cypalleles.ki.se). Of these, several have changes in the coding sequences that cause amino acid changes. Recent work in this laboratory with the expressed coding region variants indicates that most do not differ more than twofold in their kinetic parameters for several assays (phenacetin *O*-deethylation and *N*-hydroxylation of heterocyclic amines), although one of the variants (R431W) did not express holoprotein in *Escherichia coli* [299]. In cases where analysis has been done, the variations generally lead to lower activity (http://www.cypalleles.ki.se). An exception is *CYP1A2*1F* (−163 C>A), which is associated with higher inducibility. P450 1A2 is now considered to be more variable than previously thought, as evidenced by additional sites identified in an Ethiopian study [300].

Genome-wide association studies (GWAS) have identified sites in the P450 1A2 and AhR genes as being determinants for coffee consumption and induction of P450 1A2 by coffee [301–303].

9.7.2.4 Substrates and Reactions

The list of drug substrates is long [51], and only a few of the more well-known reactions are listed in Tables 9.5, 9.6, 9.7, and 9.9.

The only major endogenous substrates are 17β-estradiol and estrone (2-hydroxylation, with some 4- and 16α-hydroxylation). The physiological relevance of this reaction is unknown, particularly because of the wide variation in levels of P450 1A2 (this reaction is also catalyzed by other P450s, e.g., 3A4 [311]). Induction of P450 1A2 and 2-hydroxylation has been proposed as a means of preventing oxidation of 17β-estradiol to the potentially more reactive 4-hydroxy product [312, 313].

P450 1A2 is prominent among the human P450s involved in carcinogen bioactivation [99]. Many carcinogens are substrates, particularly aromatic and heterocyclic amines (Table 9.8, Figs. 9.9 and 9.10). Other carcinogens shown to be substrates include polycyclic hydrocarbons, nitropolycyclic hydrocarbons, and some *N*-nitrosamines [314]. One of the most relevant carcinogens is aristolochic acid, a causative agent in human nephropathy and urothelial cancer [315].

Although P450 1A2 is not generally considered to be a P450 stimulated by cytochrome b_5 [266], it has been reported that cytochrome b_5 can shift the balance of ellipticine from detoxication to bioactivation [267].

Chemical mechanisms of P450 1A2 reactions have been considered, particularly for *N*-oxygenation. A classical view involves the so-called compound I (FeO^{3+}) entity, acting via 1-electron oxidation followed by oxygen rebound [316, 317]. A deficiency of this model is that electron-withdrawing groups did not perturb *N*-oxygenation of a series of *N,N*-dimethylanilines [317], in contrast to *N*-dealkylation (which showed a negative ρ value in Hammett analysis) [317–319]. Other mechanisms have been proposed [320], including a recent "anionic" intermediate model based on theoretical studies [321].

9.7.2.5 Structure

In 2007, Johnson and his associates [271] reported an X-ray crystal structure of human P450 1A2 complexed with α-NF. That structure may be compared with the subsequently published structures of P450 1A1 [270] and 1B1 [323], which also contain the same ligand. P450 1A2 has a compact, closed active site that is appropriate for relatively large plasma molecules. In the published structure, as with P450 1A1 [270], the site of the α-NF that is oxidized (to the 5,6-epoxide is furthest away from the heme iron [214, 256]). (However, the rate of oxidation is very slow and may reflect the tendency to bind in an unproductive conformation.) The issue of cooperativity will be discussed later under P450 3A4. Cooperativity has not been reported for the human P450, but behavior of the rabbit ortholog has been interpreted in the context of multiple, overlapping binding sites [214, 324]. Docking studies suggest that two molecules of pyrene (or other small ligands) can be accommodated in the P450 1A2 site [214, 325] (Fig. 9.14).

9.7.2.6 Inhibitors

Several human P450 1A2 inhibitors are known from clinical work, including furafylline (mechanism based) [326] and fluvoxamine. α-NF is a readily commercially available and strong inhibitor of human P450 1A2 (K_I~6 nM [274]) for in vitro work. A number of polycyclic acetylenes are potent inhibitors of P450 1A2 [274]. With rat P450 1A2, TCDD and some polyhalogenated biphenyls are strong inhibitors, but these studies have not been extended to human P450 1A2 [327].

The multikinase inhibitor axitinib is also a potent inhibitor of human P450 1A2 (IC_{50} 0.1 μM) [328]. Some 7-ethynylcoumarin inhibitors have been synthesized that are selective inhibitors of human P450 1A1 and 1A2 [329]. Other ethinyl derivatives and some natural products also selectively inhibit the three human P450s in family 1 [274, 278]

9.7.2.7 Clinical Issues

Some drug interactions have been reported. An older example is that of low activity towards phenacetin favoring a potentially toxic secondary pathway, deacetylation followed by quinoneimine formation and methemoglobinemia [96]. Furafylline was a drug candidate but was never developed because of its strong P450 1A2 inhibition and interference with caffeine metabolism [330]. High levels of P450 1A2 activity have also been associated with ineffectiveness of theophylline therapy (for asthma) [331, 332].

The other concern about P450 1A2 is the same discussed earlier for P450 1A1, the cocarcinogenic effect. In this regard, there is some epidemiological evidence that high P450 1A2 activity (measured as in vivo caffeine metabolism) is associated with enhanced risk of colon cancer, although the effect was not seen in the absence of high *N*-acetyltransferase activity and high consumption of charbroiled meat [132].

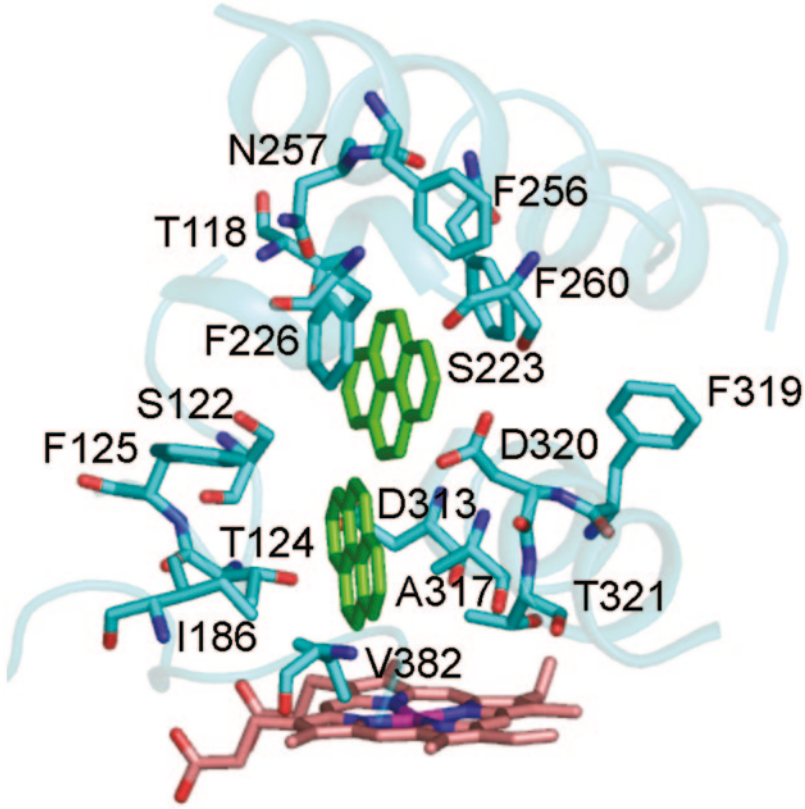

Fig. 9.14 Docking of two pyrene molecules into the active site of human P450 1A2 [214]. Pyrene molecules are in *green,* and the heme is at the *bottom* of the figure

Some evidence has been reported that P450 1A2 genetic variants can be correlated with lung cancer incidence [333].

In addition to the caffeine metabolism method of noninvasive phenotyping [61], a [^{13}C]-methacetin breath test has been reported [334].

9.7.3 P450 1B1

9.7.3.1 Sites of Expression

P450 1B1 was originally discovered in keratinocyte cultures in a search for new dioxin-inducible genes [71] and in work on adrenals in animal models [335]. In contrast to P450 1A1 and 1A2 (seven exons), the P450 1B1 gene has only three exons and is located on chromosome 2 instead of 15 [336]. Although most of the detailed studies of tissue-specific expression have been done at the mRNA level and not protein, strong responses are seen in fetal kidney, heart, and brain, in that order [259]. In adults (human), there is little detectable expression in liver but expression in kidney, spleen, thymus, prostate, lung, ovary, small intestine, colon, uterus, and mammary gland [259]. Many of these tissues are of particular interest because of the tumors that develop there. Immunochemical staining of P450 1B1 has been reported in a variety of different malignant tumors [337].

The level of expression (of the protein) in human lung has been estimated to be at the level of ~1 pmol/mg microsomal protein in nonsmokers and 2–4 pmol/mg microsomal protein in smokers, levels an order of magnitude lower than for P450 1A1 [229]. These low values may explain the lack of immunostaining in (nontumor) tissues reported by Murray et al. [337]. Specific values for levels of expression in tissues other than lung have not been published. Traces

of P450 1B1 mRNA were found in human liver using real-time polymerase chain reaction (PCR), but the protein was undetectable within the limits of sensitivity [338].

The eye is an important site relevant to the glaucoma associated with loss of activity alleles (Sect. 7.3.7, *vide infra*). In the eye (human), P450 1B1 mRNA is present at a high level in the iris and ciliary body and at lower levels in the cornea, retinal pigment epithelium, and retina [127, 339]. P450 1B1 protein is absent in the trabecular network but present in nonpigmented ciliary epithelium, corneal epithelium and keratocytes, both layers of the iris pigmented epithelium, and retina [127, 339].

P450 1B1 expression (at the protein level) has been detected in human lungs and is higher (1.8 pmol/mg microsomal protein) in smokers [229]. The level was even higher (4.4 pmol/mg microsomal protein) in ex-smokers.

It has recently been demonstrated that processing of P450 1B1 by a cytosolic serine protease activates a mitochondrial-targeting signal of P450 1B1 and leads to mitochondrial localization and activity, where functional activity results from coupling with the adrenodoxin electron delivery system [340].

9.7.3.2 Regulation

In vitro experiments show the inducibility of P450 1B1 in patterns expected for an *Ah*-responsive gene, which is one way in which the gene was found [71]. Unlike P450 1A1 and particularly P450 1A2 (*vide supra*), there is limited direct evidence for inducibility of human P450 1B1 in vivo because of the low, extrahepatic expression and the lack of a diagnostic probe drug. Although the expression of P450 1B1 is driven by the AhR system, additional factors must be involved because of the known tissue and cell line selectivity of expression. For instance, major differences are seen between HepG2, MCF-7, and ACHN cells (of liver, breast, and kidney tumor origin, respectively) [336]. With the information available today, one would expect the gene to be induced (in extrahepatic tissue) by the compounds that induce P450s 1A1 and 1A2.

In addition to the AhR regulation, the human P450 1B1 gene is also regulated by estrogens via the ER [341]. Human P450 1B1 is also regulated by microRNA [342].

9.7.3.3 Genetic Variation

Levels of P450 1B1 in human lung vary by at least one order of magnitude [229]. An interesting observation is that a termination variant of P450 1B1 is strongly associated with glaucoma [127, 343]. Other polymorphisms of (human) P450 1B1 are known and are predominantly in a set of haplotypes involving four variations, Arg/Gly-48, Ala/Ser-119, Val/Leu-432, and Asn/Ser-453. Assays involving the metabolism of 17β-estradiol and polycyclic hydrocarbons by these recombinant P450 1B1 variants show some variations but have not been particularly dramatic (reviewed by Shimada et al. [129]).

At this time, the http://www.cypalleles.ki.se website shows 26 allelic variants of P450 1B1, plus six additional ones where the haplotype has not been determined. The number of allelic variants listed in http://www.cypalleles.ki.se is an underestimate, in that many more have been reported to be associated with glaucoma (at least 82) [339]. The functional effects on some of the coding sequence variants have been determined [129, 344] but are not particularly strong (*vide supra*). There is considerable interest in genetic variations of P450 1B1 in the context of cancer and glaucoma (Sect. 7.3.7, *vide infra*).

9.7.3.4 Substrates and Reactions

Human P450 1B1 has never been purified from tissue, and all of our information has come from the protein expressed in heterologous systems. 7-Ethoxyresorufin *O*-deethylation can be used as a model reaction [345]. The catalytic activity of P450 1B1 is intermediate between P450s 1A1 and 1A2 [274]. Some other model reactions can be used as well [345].

Much of the interest in P450 1B1 has been because of its ability to activate a very broad spectrum of chemical carcinogens, including polycyclic hydrocarbons and their oxygenated derivatives, heterocyclic amines, aromatic amines, and nitropolycyclic hydrocarbons [259] (Table 9.10,

Table 9.10 Some carcinogens activated by human P450 1B1

Substrate	Reference
Polycyclic aromatic hydrocarbons	
Benzo[*a*]pyrene	[274]
Benzo[*a*]pyrene-4,5-diol	[259]
(+) Benzo[*a*]pyrene-7,8-diol	[259]
(−) Benzo[*a*]pyrene-7,8-diol	[259]
Dibenzo[*a, l*]pyrene	[344]
Dibenzo[*a, l*]pyrene-11,12-diol	[259]
Benz[*a*]anthracene	[274]
Benz[*a*]anthracene-1,2-diol	[259]
Benz[*a*]anthracene-*cis*-5,6-diol	[259]
7,12-Dimethylbenz[*a*]anthracene	[259]
7,12-Dimethylbenz[*a*]anthracene-3,4-diol	[259]
Benzo[*c*]phenanthrene-3,4-diol	[259]
Fluoranthene-2,3-diol	[259]
Benzo[*b*]fluoranthene-9,10-diol	[259]
Chrysene-1,2-diol	[259]
5-Methylchrysene	[344]
5-Methylchrysene-1,2-diol	[259]
5,6-Dimethylchrysene-1,2-diol	[259]
Benzo[*g*]chrysene-11,12-diol	[259]
6-Aminochrysene-1,2-diol	[259]
Heterocyclic amines	
MeIQ	[259]
MeIQx	[259]
IQ	[259]
Trp-P1	[259]
Trp-P2	[259]
PhIP	[259]
Aromatic amines	
2-Aminoanthracene	[259]
2-Aminofluorene	[259]
4-Aminobiphenyl	[259]
3-Methoxy-4-aminoazobenzene	[259]
o-Aminoazotoluene	[259]
6-Aminochrysene	[259]
Nitropolycyclic hydrocarbons	
1-Nitropyrene	[346]
2-Nitropyrene	[259]
6-Nitrochrysene	[259]
2-Nitrofluoranthene	[346]
3-Nitrofluoranthene	[346]
6-Nitrobenzo[*a*]pyrene	[346]
1,8-Dinitropyrene	[346]
1-Aminopyrene	[346]
Estrogens	
17β-Estradiol	[347]
Estrone	[348]

Fig. 9.10). This broad specificity of human P450 1B1 in activating aryl and heterocyclic amines, polycyclic hydrocarbons, and other carcinogens has been reviewed elsewhere [99, 259, 349–351].

Of particular interest is the observation that human P450 1B1 is at least as active as P450 1A1 in the conversion of the classic carcinogen benzo[*a*]pyrene to the 7,8-dihydrodiol, the first step in the formation of the (7,8) diol (9,10) epoxide [352]. In general, it would appear that the rodent P450 1B1 enzymes have similar catalytic specificity as human P450 towards carcinogens, from the available information [353]. If this is a valid view, then the observation that P450 1B1-knockout mice do not form tumors when administered 7,12-dimethylbenz[*a*]anthracene is of particular importance [106].

One of the interesting findings with human P450 1B1 is that this enzyme is an efficient catalyst of 17β-estradiol hydroxylation and that the pattern is for 4->2-hydroxylation [311, 347, 354]. This pattern is the opposite seen for P450s 1A2 and 3A4 (2->4-hydroxylation) [311, 355] and is of significance because 4-hydroxyestradiol is chemically more reactive with oxygen and also more likely to oxidize (to an *o*-quinone) and bind DNA [356]. Thus, 4-hydroxyestrogens are considered to be candidates for causing estrogen-dependent tumors [357]. However, mouse P450 1B1 preferentially catalyzes estrogen 2-hydroxylation compared to 4-hydroxylation, in sharp contrast to human P50 1B1 [353], providing a potentially important difference with the human enzyme. This apparent lack of conservation of selectivity has relevance in use of mouse (and rat) models in some of the biology, e.g., the human glaucoma mentioned earlier [127, 343].

9.7.3.5 Structure

Johnson and his associates [272] reported an X-ray crystal structure of human P450 1B1 bound to α-NF. The structure can be compared directly with that P450 1A2 [271] and with P450 1A1 [270] with the same ligand bound. Both P450s 1A2 and 1B1 have narrow active site cavities, explaining the preference for flat aromatic substrates. A distortion of helix F places the residue Phe-231 in position for π–π stacking with α-NF [272].

Nishida et al. [358] reported that mutagenesis of Val-395 of human P450 1B1 to Leu changed the regioselectivity of 17β-estradiol hydroxylation from the C4 position to C2, demonstrating the sensitive nature of the active site, at least with regard to some reactions. The effects of the allelic variants are probably not strong enough to be of much use in understanding the effects of those residues [129].

9.7.3.6 Inhibitors

α-NF is a strong inhibitor, as in the case of P450 1A2 [274]. Some acetylenes developed by Alworth's group have been found to selectively inhibit P450 1B1 (at least relative to P450s 1A1 and 1A2), including 2-ethynylpyrene [274]. A potential drawback to these compounds is that they are rapidly oxidized by P450 1B1.

The polyphenol resveratrol is found in red grapes and has been of interest in the context of its potential to inhibit cancer [359]. Resveratrol is a noncompetitive inhibitor of P450 1B1, with a K_I value of 23 μM in model systems [360] (with selectivity towards P450 1A1). Potter et al. [361] reported that P450 1B1 oxidizes resveratrol to the known anticancer agent piceatannol, a tyrosine kinase inhibitor. A series of methoxy-substituted *trans*-stilbene compounds of the resveratrol/rhapontigenin family were prepared and tested; of these, 2,4,3′,5′-tetramethoxystilbene was found to be a strong and selective competitive inhibitor of P450 1B1 (K_I 3 nM) and resisted demethylation [279].

Because of the roles of P450 1B1 in the activation of carcinogens [99] (Fig. 9.10), there is strategic interest in developing inhibitors of P450 1B1 [362, 363]. Another tetramethoxystilbene (2,2′,4,6′-) has been reported to be a strong inhibitor of human P450 1B1 [364], in addition to the 2,4,3′,5′-substituted stilbene [279]. A number of other compounds have been considered regarding their inhibition of P450 1B1, including derivatives of flavonoids, stilbenes, pyrenes, naphthalenes, phenanthrenes, and biphenyls [365].

9.7.3.7 Clinical Issues

No issues regarding drug interactions have been raised. The two dominant clinical issues with P450 1B1 are its potential roles in cancer and glaucoma. As with the subfamily P450 1A enzymes, an issue is that induction of P450 1B1 might increase the activation of procarcinogens (Fig. 9.10). This issue may be real, although presently there is no strong epidemiological evidence to support such a relationship. Although the coding region polymorphisms have only indicated a limited potential for contribution to cancer (*vide supra*), the evidence for its trimodal expression [122] is certainly of interest, particularly in light of the number of carcinogens that P450 1B1 activates (Table 9.10). The issue of oxidation of estrogens to reactive products is one worth considering, in light of the long-standing experimental evidence for tumorigenicity of estrogens [366]. Another matter that has only begun to be addressed is the possible metabolism of the various estrogens in postmenopausal hormone treatments (e.g., Premarin® by P450 1B1 (e.g., see [356, 367] regarding DNA adducts formed by some of these estrogens).

Because P450 1B1 has such a prominent role in carcinogen activation in vitro (Fig. 9.10) [99, 259], there is considerable interest in molecular epidemiology on the subject, as reviewed by Roos and Bolt [368]. Kamataki and his associates [122] found that the trimodal distribution of inducibility of aryl hydrocarbon hydroxylase activity (benzo[*a*]pyrene hydroxylation) is due to the induction of P450 1B1, not P450 1A1. This information is relevant to the earlier findings of Shaw and Kellerman [108, 109] correlating the inducibility with lung cancer risk in smokers. However, apparently no major progress has been reported in this area following the report of Toide et al. [122]. P450 genetic variations have been considered in the epidemiology of breast [369], head and neck [370], endometrial [371], pancreatic [372], colorectal [373], hormonal [374], and prostate [375] cancers, although overall the evidence is not strong. In a mouse model, P450 1B1 is associated with smoking-induced bone loss [376].

The other major clinical issue is glaucoma, where P450 1B1 variants are clearly associated with the disease [127, 339]. The condition is reproduced in a mouse *CYP1B1* knockout, but the mechanism is still elusive [106, 339]. As mentioned previously, P450 1B1 has a broad catalytic specificity, and estrogens, arachidonic acid, retinoids, and melatonin have all been considered as possibly being involved [339].

Finally, P450 1B1 has been considered to have a role in hypertension, possibly involving its role in arachidonic acid ω-hydroxylation [377, 378].

9.7.4 P450 2A6

9.7.4.1 Sites of Expression

P450 2A6 (formerly termed IIA3 and 2A3 [379]) was purified from human liver microsomes [17] and a cDNA was first isolated from a human liver library [380]. The protein is expressed at medium levels in liver (Fig. 9.2). In one study, the fraction of total human liver P450 attributed to P450 2A6 ranged from <0.2 to 13% among individual samples, with a mean of ~4% [52]. P450 was not found in placenta (full term) [381]. In a recent LC–MS proteomic study, P450 2A6 was found at a mean level of 63 pmol/mg liver microsomal protein, almost as high as P450 3A4 (Fig. 9.2d) [55]. However, LC–MS-determined levels were not this high in other studies [54] (Fig. 9.2b, c)

P450 2A6 is also expressed in other tissues, particularly in the nasal–pharyngeal region. Expression has been detected in nasal mucosa, trachea, lung [382], and esophageal mucosa [383]. These sites of expression are of interest regarding certain cancers. In liver cancers, overexpression of P450 2A6 protein was associated with chronic inflammation and cirrhosis [384].

P450 2A6 was found to be overexpressed in colorectal tumors [385]. P450s 2A6 and 2A13 are very similar proteins (94% identity) but differ in structure (Sect. 7.4.5, *vide infra*) and some activities, as well as localization. Both P450 2A6 and 2A13 are expressed in epithelial cells of trachea and bronchi, and only P450 2A6 (no 2A13) was detected in bronchial epithelial cells of peripheral lungs [386].

9.7.4.2 Regulation

The regulation of P450 2A6 expression has been studied in primary cultures of human hepatocytes. Expression (mRNA and protein) is inducible by rifampicin [387], phenobarbital [388], and (to a lesser extent) clofibrate, cobalt, griseofulvin, and pyrazole [388]. The nuclear receptor HNF-4 is involved in expression in cultured hepatocytes [389].

P450 2A6 transcriptional regulation has been reviewed by Pitarque et al. [390]. Induction has been shown to involve the PXR, along with PPARα [391]. P450 2A6 is also induced by estrogen via the ER [392], which may be relevant to a reported influence of the menstrual cycle on P450 2A6 activity (and the cardiovascular effects of nicotine) [393].

Other factors influencing P450 2A6 transcription are NF-Y [394] and nuclear factor-erythroid 2-related factor 2 [395]. In addition, heterogeneous nuclear ribonucleoprotein A1 has been reported to be involved in post-transcriptional regulation of P450 2A6 [396], and a polymorphism in the 3′-untranslated region affects mRNA stability and enzyme expression [397]. Finally, P450 2A6 phosphorylation has been detected in vivo [297], although the effect is not known.

9.7.4.3 Genetic Variation

At least 86 allelic variants are known, with eight haplotypes yet to be determined (http://www.cypalleles.ki.se). These include a splice variant (*12) in which *CYP2A7* exons are included and the protein has lost catalytic activity [398, 399]. Some are deletions and the activities of some of the coding region variants are known to be decreased [400]. Another SNV (*2), recognized earlier, is the L160H change which yields very low catalytic activity [401]. At least one polymorphism is important for promoter activity [402]. Also of interest is a gene deletion (*4). The incidence of these variants is racially linked [168].

In part, because of the extensive genetic variation and the metabolism of carcinogens, genetic variations have been extensively considered regarding cancer (Sect. 7.4.7, *vide infra*). P450 2A6 is involved in nicotine oxidation, and Tyndale and her group reported that individuals with low P450 2A6 activity smoke less and might have lower cancer risk [134]. This proposal seems reasonable, but the findings have been questioned. General agreement exists that defective P450 2A6 genes cause reduced nicotine metabolism (the presumed basis for reduced smoking) [403–405]. Several reports conclude that having deficient P450 2A6 reduces smoking [406–409] and also lung cancer [133, 410, 411] in smokers. The latter hypothesis has biological plausibility because many carcinogens from tobacco are activated by P450 2A6 (Table 9.8 and *vide infra*). However, other studies have not revealed any relationship between P450 2A6 genotype and smoking; cancer is also somewhat controversial [412–415]. Some of the discrepancies may be racial [416], but even this is unclear [417]. Some problems are attributed to technical shortcomings in genotype analyses [418], and a definite relationship is still lacking [418] in Caucasians but is more likely in Asians [419], where the incidence of gene deletion is higher.

9.7.4.4 Substrates and Reactions

The most characteristic and specific reaction of P450 2A6 is coumarin 7-hydroxylation [17, 380]. Coumarin 7-hydroxylation has also been used as an in vivo diagnostic assay [420–422].

Soucek [423] demonstrated that a 1:1 ratio of cytochrome b_5 to P450 was required for optimal coumarin 7-hydroxylation catalyzed by the purified recombinant enzyme. The effect of cytochrome b_5 on catalytic selectivity has not been evaluated in all reports on P450 2A6.

Coumarin 7-hydroxylation can be used in vivo with humans as a phenotypic assay. An alternative procedure is to administer caffeine to individuals and determine the conversion of 1,7-dimethylxanthine to 1,7-dimethyluric acid, a reaction catalyzed by P450 2A6 [424].

Some industrial chemicals are substrates for oxidation by P450 2A6, including alkoxyethers (used as fuel additives, e.g., *tert*-butyl methyl ether) [425] and the vinyl monomer 1,3-butadiene, a cancer suspect [426].

Some drugs are also substrates, including (+)*cis*-3,5-dimethyl-2-(3-pyridyl)thiazolidin-4-one (SM-12502) [427, 428] and tegafur [429, 430], which is converted to 5-fluorouracil. Halo-

thane is reductively converted to a free radical by P450 2A6, which can yield at least two products and initiate lipid peroxidation [431].

Some of the catalytic selectivity of P450 2A6 overlaps with that of P450 2E1 (*vide infra*). One area in which the overlap has been noted is in the oxidation of nitrosamines. P450 2A6 preferentially catalyzes the oxidation (and activation) of *N*-nitrosodiethylamine, in contrast to P450 2E1, which oxidizes *N*-nitrosodimethylamine [432, 433]. P450 2A6 is also involved in the oxidation of many tobacco-specific nitrosamines, including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [433–436]. P450 2A6 appears to be the major human P450 involved in the activation of *N*-nitrosobenzylmethylamine [437], *N*-nitrosodipropylamine, *N*-nitrosobutylamine, *N*-nitrosophenylmethylamine, and *N*-nitrosonornicotine (NNN) [438]. Fujita and Kamataki [439] studied the bacterial mutagenicity of a number of tobacco-specific *N*-nitrosamines and concluded that P450 2A6 is the major human enzyme involved in activation of all.

P450 2A6 is also involved in the metabolism of nicotine (*vide supra*). P450 2A6 is the main catalyst in the oxidation of nicotine to cotinine [440–442]. P450 2A6 is also involved in the 3′-hydroxylation of cotinine [443]. In addition, P450 2A6 catalyzes 2′-hydroxylation of nicotine, yielding a precursor of a lung carcinogen [444].

P450 2A6 can also *N*-demethylate hexamethylphosphoramide [445].

Several forms of human P450 catalyze the 3-hydroxylation of indole [446], and the product dimerizes to the dye indigo. P450 2A6 was the most active human P450 identified for this activity and could also catalyze several oxidations of indole [446]. Mutants of P450 2A6 generated from a randomized library were shown to catalyze the oxidation of several substituted indoles to generate variously colored indigos and indirubins [447].

Other substrates of interest include 1,7-dimethylxanthine, a major caffeine metabolite [448] (this can be applied in phenotyping studies), pilocarpaine [449], bilirubin [450], and metronidazole [451].

More recently, Shimada et al. [351] have demonstrated that P450 2A6 can catalyze the bioactivation of a number of PAHs and arylamines.

Yun et al. [452] analyzed the kinetics of the catalytic cycle of P450 2A6 with coumarins and concluded that substrate binding, product release, electron transfer, and oxygen binding were all rapid steps and that C–H bond cleavage is probably mainly rate limiting.

9.7.4.5 Structure

In 2005, Johnson and his associates reported the X-ray crystal structure of P450 2A6 complexed with coumarin and methoxysalen [453]. Subsequent structures with synthetic inhibitors [454] and mutants [455] added to the knowledge of this P450. It has one of the smallest active sites (~260 Å^3 volume) and is relatively rigid, although some larger ligands can be accommodated.

The structure of P450 2A6 has been compared with those of P450s 2A13 and 2E1 (with pilocarpine bound) [456] and inferences about important residues differing between these proteins have been made [457].

Some mutations, developed in random mutagenesis work [458], result in large change in the active site volume of P450 2A6 [459] (Fig. 9.15).

Lewis published several homology models of P450 2A6 and also attempted to rationalize the pattern of nicotine oxidation using molecular orbital calculations [460].

9.7.4.6 Inhibitors

Several selective inhibitors of P450 2A6 are known. Diethyldithiocarbamate appears to be a mechanism-based inactivator, although the inactivation has not been extensively characterized [433]. Diethyldithiocarbamate and its oxidized form, disulfiram, also inhibit P450 2E1 [461]. In vivo single-dose treatment of people with disulfiram inhibits P450 2E1 but not P450 2A6 [462].

A number of chemicals have been tested as inhibitors of P450 2A6 in human liver microsomes [463]. Of these, the most selective and potent inhibitors appear to be 8-methoxypsoralen, tranylcypromine, and tryptamine, with K_I values ~1 μM [463–465]. The inhibition by the natural product 8-methoxypsoralen (present in many foods) is mechanism based [466]. 8-Methoxypsoralen (methoxysalen) inhibits P450 2A6 in vivo [462] and has also been reported to decrease

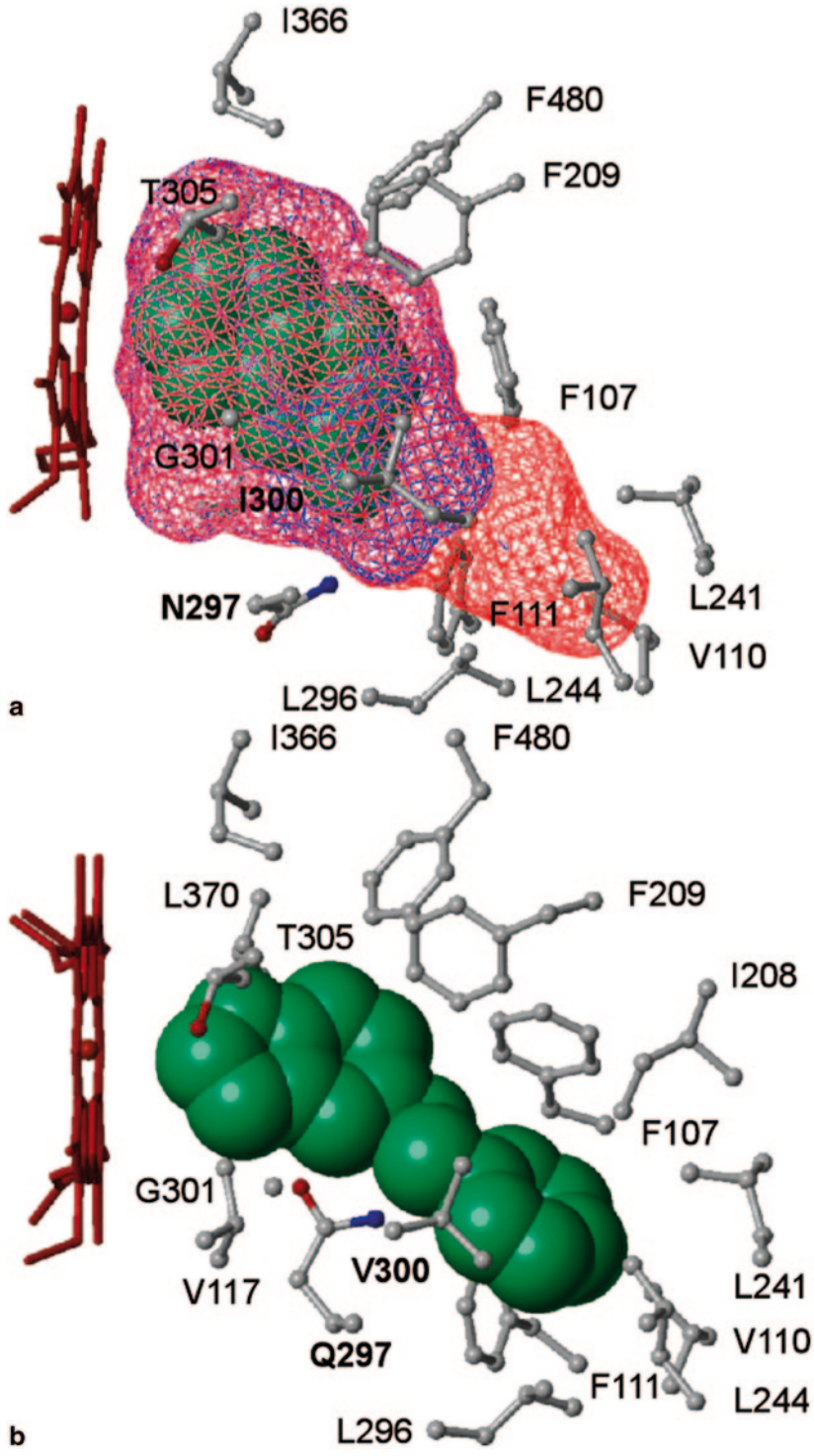

Fig. 9.15 Active site of P450 2A6. **a** In the wild-type enzyme, Ile-300 and Asn-297 restrict the available space to the area shown with the *purple* mesh (359 Å^3). The extra space made available in the I300V mutant is shown with the *orange* mesh (total 440 Å^3). **b** Minimized energy docking of the substrate 5-benzoylindole to the P450 2A6 N297Q/I300V mutant [459]

nicotine metabolism in smokers [467]. Both the inhibitors 8- and 5-methoxypsoralen were covalently bound to P450 2A6 during incubation with NADPH [468]. Menthofuran, another natural product, is also a mechanism-based inactivator of P450 2A6 [469]. Isoniazid has been reported to be a weak mechanism-based inactivator of P450 2A6 [470].

A number of heterocyclic inhibitors of P450 2A6 have been synthesized [471], and the interaction of some other new inhibitors has been visualized in P450 2A6 crystal structures [454].

The selectivity of P450 2A6 "reaction phenotyping" inhibitors was reevaluated by Stephens et al. [472], who compared chemicals for inhibition of P450s 2A6 and 2A13 (*vide infra*): tranylcypromine and (*R*)-(+)-menthofuran had > tenfold selectivity in favor of P450 2A6 > P450 2A13 and 8-methoxypsoralen had a sixfold lower K_I for P450 2A13. Khojasteh et al. [473] concluded that 3-(pyridine-3-yl)-1*H*-(pyrazol-5-yl)pyridine was more selective than tranylcypromine.

Another inhibitor of P450 2A6 is chalepensin (mechanism based) [474]. Heteroatom nicotine derivatives have been identified as inhibitors [475], and P450 2A6 is inactivated during the oxidation of nicotine itself [476].

Finally, a variety of chemicals, including PAHs, chlorinated biphenyls, and flavonoids were demonstrated to interact with (spectral binding) and to inhibit P450 2A6 (as well as P450 2A13) [477]. This inhibition has relevance to potential use of some of these compounds as therapeutic inhibitors as well as to interactions in the activation of them by P450 2A6 (and 2A13) [351].

Much of the enthusiasm about inhibitors of P450 2A6 stems from the hope of cancer prevention, in that 8-methoxpsoralen (despite the caveats about human P450 2A13 selectivity, *vide supra*) effectively deceased tumors in an NNK treatment mouse model [478].

9.7.4.7 Clinical Issues

As indicated in Sect. 7.4.2, the major issue regarding P450 2A6 polymorphisms is the effects on lung and esophageal cancers and smoking habits, for which there is epidemiological evidence in Asians [410], but reports remain controversial in Caucasians [416, 418, 419, 479, 480].

As pointed out above, some drugs are P450 substrates, although the relative contribution of P450 2A6 is still so small (Fig. 9.1b) that P450 2A6 reactions are generally not included in screens.

P450 2A6 expression has been reported to be induced during infection by (carcinogenic) liver flukes [481] and downregulated during infection by hepatitis A virus [482].

Genetic variations in P450 2A6 have been extensively considered in regard to nicotine metabolism and smoking cessation therapy [483–486], and genetic variations have been considered in the direct context of smoking-related cancers [487–490]. P450 2A6 genetic variation has also been considered in the context of hepatoxicity of coumarin [491] and pancreatic cancer [492].

9.7.5 P450 2A7

The situation involving the *CYP2A7* gene is complex, and sometimes this has even been erroneously referred to as a pseudogene [168]. Two pseudogenes (*CYP2A7PTX* and *CYP2A7PCX*) are known. The P450 2A7 mRNA transcript is produced in human liver, at roughly the same level as that for P450 2A6 [398, 493]. Gonzalez's laboratory had isolated cDNA clones now recognized as P450 2A6, the 2A6 variant L160H, and 2A7 and expressed all three in HepG2 cells [380]. Of the three, only the "wild-type" P450 2A6 incorporated heme. Others have also attempted to express P450 2A7 in heterologous systems but not reported any evidence of a catalytically active P450 2A7 holoprotein [398]. Whether or not a functional P450 2A7 is transcribed from the mRNA in human tissues is still unclear, and nothing can be said about catalytic activity.

Gene conversion events between the *CYP2A6* and *CYP2A7* genes have been reported, yielding chimeric proteins in humans [398, 399, 494]. These proteins have some of the coumarin 7-hydroxylation conferred by the 2A6 component [399].

It has been reported that there are at least four polymorphic P450 2A7 gene variants, and some of these can be confounding when genotyping for certain P450 2A6 alleles [495].

9.7.6 P450 2A13

9.7.6.1 Sites of Expression

P450 2A13 cDNA was first cloned from a human nasal mucosa library [445]. mRNA was detected primarily in nasal mucosa, trachea, and lung, with the level in liver being only ~1% of that in nasal mucosa [496]. This is in sharp contrast to P450 2A6, which is primarily a liver enzyme (see Sect. 7.5.1, *vide supra*). At the protein level, immunochemical analysis has shown P450 2A13 in the epithelial cells of human bronchus and trachea [386, 497]. P450 2A13 has also been detected in human bladder [498], and there are reports of some expression in brain, mammary gland, prostate, testes, and uterus [496] and pancreatic α-islet cells [499].

P450 2A13 mRNA was reported to be elevated in small-cell lung cancer tissue in one study [500] but was not detected or was downregulated in any lung cancers in two other studies [497, 501].

9.7.6.2 Regulation

P450 2A13 transcription involves CcATT/enhancer-binding protein (C/EBP) transcription factors [502]. This interaction is believed to be responsible for olfactory mucosa-specific expression in humans. In addition, there is evidence for epigenetic regulation of P450 2A13 expression, at both the levels of DNA methylation and histone acetylation [502, 503].

9.7.6.3 Genetic Variation

At least 21 different CYP2A13 gene variants have been reported (http://www.cypalleles.ki.se). There is evidence that some of those that produce amino acid changes can alter catalytic properties and that expression levels can change [504–508]. Genetic differences are racially linked [503, 509–511].

9.7.6.4 Substrates and Reactions

Although P450 2A13, 94% identical to P450 2A6, can oxidize some relatively common subfamily 2A P450 substrates such as coumarin [512], the interest in P450 2A13 has been driven by its ability to activate procarcinogens [496]. The catalytic efficiency in activating the so-called tobacco-specific nitrosamines (NNK, NNN) is considerably higher than P450 2A6. When coupled with the selective expression of P450 2A13 in the respiratory tract, there is potential for understanding aspects of tobacco-induced cancers of the lung and the rest of the respiratory tract [496, 513].

The active site of P450 2A13 is larger than that of P450 2A6 (*vide infra*), and a number of additional substrates of P450 2A13 have been identified, including nicotine and cotinine [514], the nicotinium Δ^5 (1′) iminium ion [515], aflatoxin B_1 [516, 517], phenacetin and theophylline [518], 4-aminobiphenyl [498], and 5-methoxypsoralen [519]. In addition, P450 2A13 was found to activate a large variety of PAHs (and their dihydrodiol derivatives), arylamines, and heterocyclic amines to genotoxic products [351].

The relevance of the activation of all of these procarcinogens can be addressed in a P450 2A13-humanized mouse model [520].

9.7.6.5 Structure

Some early site-directed mutagenesis work implicated roles of certain amino acids in the metabolic activation of NNK [521]. A structure of P450 2A13 was reported by Scott's laboratory in 2007 [522]. Although no substrate had been added, the finished structure revealed the presence of indole, which is known to be a substrate. Like P450 2A6, the active site cavity is relatively small and hydrophobic, with a cluster of Phe residues composing the roof. The size of the active site appears to be larger than that of P450 2A6. Residues at positions 117, 300, 301, and 208 help define differences with P450 2A6 [522]. Some computational work has also appeared [523].

Another structure has been reported with pilocarpine (an imidazole) bound [456]. As might be expected from the imidazole ring and the type

II binding spectrum, the imidazole ring was closest to the heme (the Fe–N distance was 2.3 Å). The pilocarpine-bound structure was compared to that of P450 2A6 and 2E1 [456].

9.7.6.6 Inhibitors

In light of the activation of procarcinogens by P450 2A13, there is interest in developing inhibitors to prevent cancer, and some success has been achieved [471, 524–526]. One of the issues is selective inhibition of P450 2A13 relative to P450 2A6. Some compounds, e.g., 8-methoxypsoralen, menthofuran, and β-tyramine, show an order of magnitude selectivity for P450 2A13 > P450 2A6 [472, 527, 528]. Nicotine is a mechanism-based inactivator of P450 2A13 [476]. Shimada et al. [477] examined 66 chemicals as inhibitors of P450 2A13, including a variety of flavonoids and polycyclic hydrocarbons. Several selectively inhibited P450 2A13 (relative to P450 2A6), with low- or sub-μM IC_{50} values. One of the conclusions, based upon spectral binding studies, was that the active site of P450 2A13 is more spacious than that of P450 2A6, consistent with the X-ray crystal structure (*vide supra*).

9.7.6.7 Clinical Issues

P450 2A13 can oxidize some drugs [518], but there is no evidence that it makes a major contribution to the clearance of any (Fig. 9.1b). The major issue is possible contribution to cancers of the respiratory tract, particularly those caused by smoking [496]. Accordingly, a number of epidemiological studies have been done, particularly with regard to alleles associated with lower metabolic activity [503, 505, 507, 529–533], with at least some of the studies showing significant correlations of lung cancer with risk in smokers associated with P450 2A13 genotypes [503, 505].

9.7.7 P450 2B6

9.7.7.1 Sites of Expression

P450 2B6 is expressed primarily in liver, and the protein was partially purified [534]. The protein has also been detected in human lung [535].

Much of the early work with P450s in experimental animals was focused on the phenobarbital-inducible enzymes now recognized to be in the P450 2B subfamily [536, 537] and a general expectation was that similar P450s would be prominent in human liver (and further suggested by immunochemical studies [9] and early cloning work [538]). However, the major P450 in human liver (and small intestine) proved to be P450 3A4 (Figs. 9.2 and 9.3). The mean level of P450 2B6 in human liver has been somewhat controversial.

One of the problems has been antibody specificity. Antibodies raised against rat P450 2B1 have not been very specific [534]; unfortunately many papers in this area show only limited sections of gels (or actually show major cross-reactive material present). The results tend to fall into two groups. One set reports levels vary from low to 80 pmol P450 2B6 per mg protein [539–541]. Another set of reports ranges from near-zero levels to 28 pmol P450 2B6/mg microsomal protein [534, 542–545]. However, the mean values differ considerably in the former and latter groups. While some of the discrepancy may be attributable to the differences in liver samples, the main difference may be with the antibodies used and cross-reactivity with other proteins, as well as error inherent in other aspects of immunochemical methods. Our own work is in line with the lower set of estimates of expression levels (mean 1–2 % of total P450, with values rarely exceeding 5 % even in samples from individuals administered inducers) [545]. This level is an order of magnitude less than for P450 3A4 (Fig. 9.2).

Recently Achour et al. [55] used an LC–MS proteomic approach with human liver microsomes and reported a mean value of 39 pmol/mg protein. This value was ~one half of that found for P450 3A4 in the same set of samples (Fig. 9.2d). The concentration was much less in the other samples (Fig. 9.2a, b, c), with another LC–MS study reporting only 0.5 and 7 pmol P450 2B6/mg protein [54].

9.7.7.2 Regulation

Studies with HepG2 cells (derived from hepatocytes) have shown the role of CAR, a member

of the steroid receptor superfamily, and its interaction with the phenobarbital-responsive enhancer module (PBREM) in the region between −1733 and −1683 bp in the 5′-flanking region [546]. Other work with HepG2 cells implicated the liver-selective transcription factor C/EBP*α* [547]. Kliewer's group [548] also demonstrated the involvement of another previously orphan receptor, PXR, in binding to PBREM in primary human hepatocytes to induce P450 2B6. PXR is active only when ligand activated, but CAR apparently acts without an added ligand; both CAR and PXR heterodimerize with (liganded) RXR [549] (Fig. 9.13). "Cross talk" also exists at the PBREM site with the vitamin D receptor (VDR) as well as CAR and PXR [550, 551]. The levels of CAR and PXR mRNA in individual human livers correlate with the level of P450 2B6 mRNA [552]. The regulation of P450 2B6 has considerable similarity to those of the P450 2C and 3A subfamilies (*vide infra*), with some differences. CAR does have ligand-activated effects and 6-(4-chlorophenyl)imidazo[2,1-*b*;1,3] thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl) oxime has been identified as an agonist [553]. A novel distal enhancer regulated by PXR and CAR was identified in the *CYP2B6* gene [554].

The roles of nuclear receptors in P450 2B6 induction have been reviewed by Wang and Negishi [555] and Wang and LeCluyse [556]. In primary human hepatocytes, P450 2B6 was induced by clotrimazole, phenobarbital, rifampicin, ritonavir, carbamazepine, and phenytoin, with all but the latter two compounds apparently activating via PXR [557].

Negishi's group also reported a novel CAR-mediated mechanism for synergistic activation of two distinct elements within the P450 2B6 gene [558]. Neurosteroids and nicotine were identified as PXR activators [559]. Negishi and his associates were able to classify P450 2B6 inducers in terms of PXR versus CAR mechanisms [560]. They also showed that CAR was an early growth response factor in activating the P450 2B6 gene [561]. Oltipraz, generally considered in the context of Nrf2, also activates CAR [562]. Metformin represses P450 2D6 by modulating CAR signaling [563].

P450 2B6 has been found to be phosphorylated in vivo, although the effect on activity is unknown [297].

9.7.7.3 Genetic Variation

As mentioned in the previous edition of this chapter [149], P450 2B6 is highly polymorphic. At least 63 allelic variants have been identified, and at least six more variants are known in which the haplotypes have not been determined yet (http://www.cypalleles.ki.se). A number of these are known to be associated with lower activity, and a number of clinical consequences have been reported (Sect. 7.7.7, *vide infra*). A partial deletion of the P450 2B6 gene has been attributed to crossover with the pseudogene *CYP2B7* [564].

9.7.7.4 Substrates and Reactions

The number of P450 2B6 substrates has grown with time but is still not as extensive as for P450 3A4 (Sect. 7.20.4, *vide infra*). However, with the availability of more knowledge about genetic variants (*vide supra*) and diagnostic marker substrates, it has been possible to show the relevance of P450 2B6 in vivo in several cases.

The substrate specificity of P450 2B6 has been reviewed [565–567]. "Marker" fluorescent substrates are available for some in vitro uses [568].

One diagnostic substrate is efavirenz [569], which also has clinical issues (Sect. 7.7.7, *vide infra*). Perhaps the most widely accepted reference substrate for P450 2B6 (in vitro) is bupropion [570, 571]. Efavirenz has been utilized as a marker in vivo [572].

An important substrate for P450 2B6 is the antimalarial drug artemisinin [573]. Another is methadone, used in treating heroin addiction [574]. P450 2B6 is also involved in the metabolism of a number of environmental chemicals, including the pesticide chlorpyrifos [575].

9.7.7.5 Structure

Relatively little site-directed mutagenesis has been done with P450 2B6. Halpert's laboratory modified ten residues and measured some activities, although most of the changes were ≤twofold [576]. Halpert and his associates have published

several X-ray crystal structures of P450 2B6, some with inhibitors [577–579] and one with a substrate, amlodipine [580] (this is also a substrate for P450 3A4). Several features are of note. The apparent size of the active site is large but not as large as that of P450 3A4 or P450 2C9. Two amlodipine molecules are bound in the enzyme structure. Finally, the protein is malleable and residues move to accommodate different ligands.

Several homology models of P450 2B6 have been published [581, 582], including one using molecular dynamics [583].

Yamazaki and his associates have published a two-dimensional model for rationalizing and predicting substrates for P450 2B6 [584]. In silico approaches have been used for the prediction of P450 2B6 substrates [585].

9.7.7.6 Inhibitors

A list of the reported inhibitors of P450 2B6 has been compiled by Rendic [51]. Orphenadrine had been utilized in some work with microsomes but does not appear to be particularly selective [586, 587]. 2-Isopropenyl-2-methyladamantane and 3-isopropenyl-3-methyldiamantane have been reported as selective inhibitors of P450 2B6 [588]. Triethylenethiophosphoramide has also been reported to be a selective inhibitor of P450 2B6 [589].

Khojasteh et al. [473] reported that 2-phenyl-2(1-piperidinyl)propane is the most selective in vitro inhibitor for use in reaction phenotyping. New inhibitors have been considered based on structure–activity relationships [590]. The effect of a K262R substitution on inhibition by several drugs has been noted by Hollenberg and his associates [591], and Thr-302 has been implicated (by the same group) in irreversible inactivation by *tert*-butylacetylene [592].

The oral contraceptive 17α-ethinylestradiol is a mechanism-based inactivator of P450 2B6 and modifies the (apo) protein [593, 601], but the in vivo relevance of the inhibition has not been established. Inhibition by duloxetine has been described as being both reversible and time dependent [594]. Other P450 2B6 inhibitors include an acetylenic drug candidate [595], ticlopidine, clopidogrel [596, 597], phencyclidine [598], diaziridines [599], peroxynitrite [600], methadone [602], selegiline [603], sibutramine [604], and ritonavir [605]. Another clinically relevant inhibition involves grapefruit juice [606].

9.7.7.7 Clinical Issues

Some of the clinical issues have been reviewed recently by Zanger and Klein [607]. The major issues are interindividual variations due to induction and genetic variation as well as some inhibitions. The effects of genetic variation have been reported for the drug efavirenz (used for HIV) [608–611]. Another drug in which genetic variations make an in vivo difference is bupropion, used in smoking cessation therapy [612, 613]. Efavirenz–bupropion interactions have also been reported [614].

Genetic variations have not been found to effect nicotine metabolism (or plasma levels) [615, 616]. However, genetic variations in P450 2B6 have been associated with the outcome of cyclophosphamide therapy [617–619] and the doses of methadone used in addiction therapy [620]. Other drugs for which genetic variations have been shown to be important are sibutramine [621] and imatinib [622]. Genetic variation has also been reported to contribute to the metabolism of the insecticide chloropyrifos [623].

The phenomenon of barbiturate-like enzyme induction is still an issue in drug development, however. The point is not only drug interactions but particularly the prospect of tumor promotion in rodent cancer bioassays, which is probably unrelated to the P450 induction [138].

9.7.8 P450 2C8

The P450s in the 2C subfamily have been of interest for some time. Some of the first human P450 preparations purified were probably P450 2C9, in retrospect [9, 10]. A major impetus for research in this field was the observed genetic polymorphism in (*S*)-mephenytoin 4′-hydroxylation [624, 625], which led to efforts at purification. Purified proteins had some catalytic activity towards mephenytoin [15], but subsequent in vivo pharmacokinetic [626] and heterologous expres-

sion experiments [627] demonstrated a distinction between tolbutamide and (*S*)-mephenytoin hydroxylation. Genomic analysis indicated the complexity of the *CYP2C* gene subfamily [628]. Subsequently the subfamily was characterized in terms of four P450s: 2C8, 2C9, 2C18, and 2C19 [629]. P450 2C19 is the polymorphic (*S*)-mephenytoin 4′-hydroxylase [22, 630]; P450 2C9 is involved in a considerable number of drug oxidations (Fig. 9.3). Two previous entries in the P450 nomenclature, 2C10 and 2C17, are considered allelic variants of other genes or other artifacts and have been deleted [631].

9.7.8.1 Sites of Expression

P450 2C8 was first purified from human liver [15]; the enzyme is known to be expressed in liver and kidney [632]. The level of expression of P450 2C8 has been estimated at 11–29 pmol/mg in liver microsomes using LC–MS [54, 55] but may be one of the more substantial P450s in the kidney. Other sites of P450 2C8 (mRNA) include adrenal gland, brain, uterus, mammary gland, ovary, and duodenum [633]. Expression has also been detected in cardiovascular tissue [634].

Proteomic analysis of human liver indicated P450 2C8 was detected in all samples analyzed [54, 55, 635]. A lack of effect of gender, age, or genotype on expression has also been reported [636].

Kemper and his associates have presented evidence that P450 2C8 exists as a dimer in membranes [637]. Avadhani and his associates have reported that a significant fraction of P450 2C8 is localized and functionally active in mitochondria [638].

9.7.8.2 Regulation

The level of P450 2C8 expression in human liver varies at least 20-fold [54, 55, 639]. Rifampicin induces P450 2C8 in hepatocyte culture [387]. The enzyme appears to be inducible by barbiturates [640]. Transcriptional regulation involves the nuclear receptors CAR, PXR, HNF-1α, and the glucocorticoid receptor [641].

As mentioned earlier, P450 2C8 has reported to be phosphorylated in vivo [297], but the effect on catalytic activity is unknown. Post-transcriptional control of P450 2C8 by microRNAs 103 and 107 has been reported in human liver [642].

An interesting approach with the inhibitor gemfibrozil has been used to estimate the (human) in vivo half-life of P450 2C8 at 20 h [643].

9.7.8.3 Genetic Variation

The http://www.cypalleles.ki.se website currently lists 16 allelic variants of P450 2C8. The functional effects of eight of these have been reviewed by Totah and Rettie [644]. For in vitro studies on the functional effects of P450 2C8 variations, see [645, 646]. Two coding region polymorphisms involve the amino acid substitutions I264M and K399R, with the latter appearing in a haplotype with R139K [639]. The rate of oxidation of taxol (paclitaxel) is decreased with the *3 allele (K399R/R139K), but the extent of the decrease has been variable in different studies, ranging from 90% [632] to 25% [639, 647]. The *1C polymorphism appears to cause some attenuation of the mean level of expression [639]. In vivo clinical effects of P450 2C8 variants have been reviewed by Daily and Aquilante [648], and the results are not always consistent with in vitro studies.

P450 2C8 variants show racial linkage [644, 649].

Some of the drugs considered for response (in metabolism) in regard to genetic variation include rosiglitazone [650, 651], amiodarone [652, 653], and paclitaxel and 13-*cis*-retinoic acid [654].

9.7.8.4 Substrates and Reactions

P450 2C8 does not appear to have the general significance of P450 2C9 or 2C19 in drug metabolism (Fig. 9.1b). An important substrate is taxol (paclitaxel)(6α-hydroxylation) [152, 655]. Another substrate for P450 2C8 is all-*trans*-retinoic acid [656]. P450 2C8 also contributes to the oxidation of troglitazone [657] and verapamil, rosiglitazone, cerivastatin, amiodarone, dapsone, and amodiaquine [51, 639].

In general, P450 2C8 has relatively low catalytic activity towards the known substrates of P450s 2C9 and 2C19. However, Mansuy and his associates have synthesized model substrates that all of the human subfamily 2C P450s have activity towards [658, 659].

The substrates of P450 2C8 have been reviewed by Totah and Rettie [644] and more recently by Niwa and Yamazaki [660]. P450 2C8 is involved in the oxidation of pioglitazone [661], repaglinide [662], montelukast (now considered a "classic" P450 2C8 ligand) [663, 664], an endothelin ET_A receptor antagonist ((*H*)-(5*S*,6*R*,7*R*-2-isopropylamino-7-[4-methoxy-2-[(2*R*)-3-methoxy-2-methylpropyl]]-5-(3,4-methylenedioxyphenyl)cyclopenteno(1,2-*b*)pyridine 6-carboxylic acid)) [665], imatinib [666], and 4-hydroxyretinoic acid [667]. P450 2C8 has been assigned major roles in the metabolism of amiodarone, amodiaquine, arachidonic acid, cerivastatin, chloroquine, paclitaxel (taxol), repaglinide, retinoic acid, tazarotenic acid, and troglitazone [644].

Molecular differences in genetic variants regarding several probe substrates have been considered in the context of binding affinity [668].

9.7.8.5 Structures

An X-ray crystal structure of P450 2C8 was published by Johnson and his associates in 2004 [669]. This structure is of interest in that two molecules of palmitic acid, derived from the bacteria (used for heterologous expression), were bound to the dimer interface. Another series of structures from the Johnson group [223] were solved with montelukast, troglitazone, felodipine, and 9-*cis*-retinoic acid present. The size of the active site is large (~1400 $Å^3$), similar to that of P450 3A4 (*vide infra*), but more rigid, with an "L-shape." In the case of 9-*cis*-retinoic acid, a second molecule was located above the proximal ligand and is postulated to "push" the first for more efficient oxygenation (although no evidence for binding or catalytic cooperativity was found) [223]. There is flexibility in the active site, and the ability of Arg-241 and other residues to reorient was noted.

A gating mechanism has been proposed for P450 2C8 based on theoretical studies [670].

9.7.8.6 Inhibitors

In contrast to P450 2C9, sulfaphenazole is not a strong inhibitor of P450 2C8. Mansuy's group synthesized several sulfaphenazole-based selective inhibitors of individual P450 2C enzymes, including P450 2C8 [671, 672]. Early work on paclitaxel metabolism suggests that high concentrations of the natural flavonoids naringenin, quercetin, and kaempferol and the synthetic α-NF inhibit [152], but little in vivo inhibition would be expected. Walsky et al. [673] have screened 204 drugs for P450 2C8 inhibition. P450 2C8 inhibitors have also been reviewed by Totah and Rettie [644] and Niwa and Yamazaki [660]. One of the most useful diagnostic inhibitors is montelukast, a leukotriene receptor antagonist that has also been used in a crystal structure (Sect. 7.8.5, *vide supra*) [223] and has clinical significance [674]. Another selective inhibitor reported recently is the tyrosine kinase inhibitor nilotinib [675].

Another selective inhibitor with clinical significance is the fibrate gemfibrozil. The mechanism is unusual in that the glucuronide conjugate is oxidized in the (large) active site of P450 2C8, leading to irreversible inactivation due to heme alkylation [676–678].

9.7.8.7 Clinical Issues

Some of the current issues have been reviewed by Totah and Rettie [644] and Niwa and Yamazaki [660].

Induction and inhibition of P450 2C8 are not major issues at this point (Tables 9.6 and 9.7). The epoxides formed from arachidonic acid (epoxyeicosatrienoic acids or "EETs") by P450 2C8 (and 2C9) have been considered in cardiovascular protection and in cancer therapy [679]. However, no disease etiology with P450 2C8 has been implicated at this point. The most serious issue is probably any impact on the disposition of the cancer chemotherapeutic agent paclitaxel. Polymorphisms may have some effect on in vivo 6α-hydroxylation [632, 639], although any influence may be modulated in part by the contribution of P450 3A4 to other reactions [152].

One issue was the statin (3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitor) cerivastatin, which was withdrawn shortly after marketing due to rhabdomyolysis issues. This drug had several issues, but some are related to it being a P450 2C8 substrate. Two problems were the interaction with the fibrate

gemfibrozil (*vide supra*) [680] and the influence of genetic variations of P450 2C8 [681–683].

Genetic variations in P450 2C8 have been related to amodiaquine efficacy in malaria treatment [684], response to paclitaxel treatment for breast cancer [685], pioglitazone pharmacokinetics [686], and celecoxib pharmacokinetics [687].

9.7.9 P450 2C9

In retrospect, many of the observations regarding in vivo metabolism of barbiturates [2, 688] are some of the first reports on what is now known as P450 2C9. P450 2C9 is one of the major enzymes involved in drug metabolism (Fig. 9.1b). Some of the first purified human liver P450s can now be recognized as P450 2C9 [9, 10]. A protein purified with some mephenytoin 4′-hydroxylation activity ($P450_{MP\text{-}1}$) was also P450 2C9 [15], and the cDNA corresponded to the N terminus determined by Edman degradation [689]. Proteins now recognized as P450 2C9 were also purified from liver on the basis of their oxidation of tolbutamide [626] and hexobarbital [690, 691]. The human P450 2C subfamily is complex [628], and characterization of individual members was not achieved without heterologous expression and careful analysis of catalytic activities [627, 692]. A transcript designated as P450 2C10 from this laboratory had only two coding region changes [628]. This is now recognized as an allelic variant of P450 2C9; the original assignment had been based on the unexplained distinct 3′ noncoding sequence [628].

9.7.9.1 Sites of Expression

P450 2C9 is primarily a liver P450. The hepatic level of expression is probably the highest, on the average, except for P450 3A4 (Figs. 9.2 and 9.6) [52].

All subfamily 2C P450 enzymes are expressed at only low levels in fetal liver, including P450 2C9 [689], and levels rise quickly in the first month after birth [693]. Very low levels of P450 2C9 (1–2% of adult values) were detected during the first trimester in fetal livers with values rising to ~30% in the second and third trimesters [694]. Pharmacokinetic experiments with accepted P450 2C9 substrates indicate that the level of hepatic P450 2C9 does not change with age, at least to 68 years [695].

P450 2C9 is also expressed in the small intestine (Fig. 9.3) [696]. P450 2C9 has also been detected in aorta and coronary artery [634], which may have relevance to hypertension and other cardiovascular disease.

In human (adult) liver microsomes, P450 2C9 is one of the most plentiful P450s, usually following only P450 3A4. One LC–MS proteomic analysis gave a mean of 40 pmol P450 2C9/mg microsomal protein (range 17–139) [55]. Another analysis [54] reported 80 pmol P450 2C9/mg microsomal protein for a pooled sample and a mean of 28 pmol P450 2C9/mg microsomal protein (range 8–61) for another set (Fig. 9.2).

9.7.9.2 Regulation

Early work with human hepatocytes showed induction of P450 2C9 by barbiturates and rifampicin [697], consistent with earlier in vivo work on the induction of barbiturate metabolism [688]. Subsequent studies have shown that P450 2C9 expression is induced by rifampicin, dexamethasone, and phenobarbital in hepatocytes [640, 698]. The induction involves the glucocorticoid receptor, CAR, and PXR, with CAR and PXR apparently competing at the same site [699].

Recently evidence for action of CAR at an additional site has been presented [700]. It should be emphasized that the action of CAR is somewhat different than that of other receptors from the steroid superfamily, in that it enhances transcription in the absence of a bound ligand and some of the control is at the level of nuclear translocation related to dephosphorylation of Thr-38 [179, 701]. Other factors involved are HNF-4 [702] and C/EBPα [547], accounting at least in part for hepatic localization.

The P450 2C9 promoter contains several regulatory elements, including two HNF-4α sites, a PXR site, a CAR site, and a glucocorticoid response element [703, 704]. In addition, GATA-4 [705] and ER α [706] regulation have been reported.

9.7.9.3 Genetic Variation

The genetic polymorphism of P450 2C9 has been studied extensively and has major clinical significance, although P450 2C9 has not been shown to have a critical function in normal physiology. Tolbutamide metabolism had been reported to display polymorphism [707], which was an impetus to purify the protein catalyzing the hydroxylation [626]. At least 65 alleles are known, plus eight SNPs that have not been classified as to haplotype (http://www.cypalleles.ki.se). Some of these are in the promoter region and have functional consequences for drug therapy, e.g., phenytoin [708]. Two of the most studied polymorphisms are *2 (R144C, *rs1799853*) and *3 (I359 L, *rs1057910*). Both have much lower frequencies in Asians and Africans [703]. A six-base deletion in the coding region lowered catalytic activity in a recombinant enzyme [709]. A number of P450 2C9 SNVs have been identified [710] and their racial linkage has been explored [711]. Of some interest, in addition to the *2 and *3 alleles with generally lower catalytic activity, is the *5 allele (of higher frequency in Africans) with lower catalytic activity [712]. Some of the SNPs occur in the 5′-flanking region and attenuate the expression of P450 2C9 [713]. Also of interest is an unusual phenomenon in which the *CYP2C18* exon 1-like locus is fused with combinations of exons and introns from *CYP2C9* to yield chimeric RNA transcripts [714]. Finally, linkage between *CYP2C8* and *CYP2C9* genetic polymorphisms has been reported [715].

The functional difference of 36 (protein) variants was analyzed in vitro and showed a 100-fold variation in the catalytic efficiency towards losartan (k_{cat}/K_m) [716]. Another study analyzed the functional effects of 32 variants with warfarin and tolbutamide, also reporting a variation of at least two orders of magnitude [717].

It has long been known that the functional effect of a genetic variant in (the coding region) of P450 2C9 is substrate dependent, which is not surprising in light of our current understanding of P450 function [718].

Some unusual variants are those involving promoter variations [708], and a splice variant with a ten-residue section substituted for the normal 18 residues near the N terminus gave a typical $Fe^{2+}\cdot CO$ versus Fe^{2+} difference spectrum but no catalytic activity [719].

The reason for the lower activities of the common *2 and *3 variants has been considered. One report has attributed the effect to changes in uncoupling [720]. Our own work, using arachidonic acid as a ligand, indicates that the difference can be explained simply by rates of reduction of P450 2C9, the step which appears to be rate limiting [721].

9.7.9.4 Substrates and Reactions

P450 2C9 is one of the major P450s involved in drug metabolism (Fig. 9.1b). Some earlier aspects of substrate specificity were reviewed by Miners and Birkett [722] and by Rendic [51]. One of the early substrates examined was phenytoin, which undergoes 4-hydroxylation [15]. P450s 2C18 and 2C19 can also catalyze this reaction, but P450 2C9 is the major catalyst [723].

Mansuy's group used the P450 2C9 inhibitor sulfaphenazole to build a substrate common to all four subfamily 2C P450 enzymes [658].

Some compounds normally in the body are oxidized by P450 2C9, including arachidonic and linoleic acids (epoxidation) [724] and vitamin A (all-*trans*-retinoic acid, 4-hydroxylation) [725], although the physiological significance is unknown. P450 2C9 oxidizes arachidonic acid to several of the epoxides (EETs), which have important vascular and other properties [726–731].

Several reactions have been used as in vivo probes, including tolbutamide, warfarin, flurbiprofen, and losartan [732].

One substrate of recent interest is celocoxib, a cyclooxygenase-2 inhibitor (Celebrex®). P450 2C9 is the major catalyst of oxidation, and variants affect the in vivo pharmacokinetic parameters [733, 734].

Several aspects of P450 2C9 reactions are of concern regarding interpretation of results, at least in in vitro research. One issue is the effect of solvents on catalytic activity [735]. A concentration of 1% (v/v) CH_3CN markedly inhibited the catalytic activity of P450 2C9 [735]. Another issue is the enhancement of most reactions by cytochrome b_5 [266]. Further work also showed

that apo-cytochrome b_5 (devoid of heme) was as effective as cytochrome b_5 [736], arguing against a need for electron transfer. Other work showed that even other P450s could enhance the rates of some P450 2C9 reactions, even though those P450s did not catalyze the reactions themselves [266]. These results are reminiscent of some of the interactions of rabbit P450s 1A2 and 2B4 reported by Backes [737].

Other work with P450 2C9 has provided evidence for cooperativity in some reactions, although the area has not been as developed as for P450 3A4 (*vide infra*). Dapsone and some analogs enhance the binding and 4-hydroxylation of diclofenac [738, 739]. However, the activity of P450 2C9 towards dapsone is unaffected by diclofenac, in a situation similar to that of P450 3A4, aflatoxin B_1, and α-NF [740]. The interpretation that P450 2C9 uses two binding sites in these interactions is probably valid [739], although (as with P450 3A4) the mechanism remains to be elucidated (including the exact nature of the binding).

The substrates for P450 2C9 have been reviewed by Niwa and Yamazaki [660] and compared with the other three subfamily 2C P450s. Important drugs that are oxidized (mainly) by P450 2C9 include irbesartan, losartan, phenytoin, cyclophosphamide, tamoxifen, fluvastatin, celocoxib, diclofenac, ibuprofen, lornoxicam, meloxicam, naproxen, glibenclamide, glipizide, tolbutamide, and warfarin [703]. A list of the drugs for which genetic variation in P450 2C9 has been an issue in clinical practice has also been published (Table 9.5) [741]. For in vitro work, tolbutamide and diclofenac are considered the most validated substrates [742]. Tolbutamide, recognized early as a substrate [626], is also used for in vivo phenotyping [743].

P450 2C9 contributes to the 2-hydroxylation of the oral contraceptive 17α-ethinylestradiol [744]. Another substrate is nabumetone [745].

Some compounds are activated to potentially dangerous electrophilic products, including the natural product safrole [746] and two drug-related thiophenes [747].

9.7.9.5 Structure

Two important X-ray crystal structures have been published, one with bound warfarin [41] and one with flurbiprofen [748]. The active site is relatively large, allowing many drug substrates, and Arg-108 is involved in binding to the carboxylates of some of the substrates [748]. The structure has been compared with those of P450 2C8 and 2C9 [749].

The importance of Arg-108 has been underscored by site-directed mutagenesis studies [750], although the picture is more complex than simple substrate charge pairing. The roles of other residues have also been studied by site-directed mutagenesis, including Phe-114, Phe-476, and Leu-208 [751]. Movement of the helix B–C loop and Arg-108 between the open and closed (substrate bound) forms has been proposed [749].

Theoretical studies have been done on P450 2C9 protein dynamics and substrate binding [752, 753]. Structures and other information have been utilized to develop models for the prediction of substrate binding and reactivity [754–758].

Changes in particular residues of P450 2C9 yield markedly different effects depending on the substrate and reaction under consideration. For instance, the polymorphism *3 (I359 L), which appears to be very conservative, changed catalytic efficiencies of different reactions by factors of 3- to 27-fold (in vitro) [759]. Although the *2 and *3 polymorphisms cause considerable changes with some substrates, diclofenac metabolism is not altered [760], consistent with the in vitro findings.

With the above caveats, the roles of a number of amino acids have been examined with several reactions, although extrapolation to more reactions requires caution. Changes in Arg-97 and Arg-98 affected activity towards diclofenac [761]. Asp-293 has been shown to have a relatively general structural role, possibly by bonding to a partner amino acid or amide [762]. Studies with coumarins suggested two sites, one for π-stacking of aromatic rings and an ionic binding site for organic anions [763]; many P450 2C9 ligands have an anionic charge [764, 765].

P450 2C9 was converted into an enzyme with (*S*)-mephenytoin 4′-hydroxylation activity (i.e., P450 2C19-like) with a relatively small number of changes (I99H, S220P, P221T, S286N, V292A, F295 L). Conversely, P450 2C19 could be transformed to an enzyme with warfarin hydroxylation activity similar to that of P450 2C9 (and also sulfaphenazole binding) with the changes N286S, I289N, and E241K [766]. Mansuy's laboratory identified residues 476, 365, and 114 as being important in diclofenac and sulfaphenazole binding and in inactivation by tienilic acid [767]. Phe-114 is proposed to be involved in π-stacking [767], perhaps serving the role proposed in the coumarin studies mentioned earlier [763].

9.7.9.6 Inhibitors

Sulfaphenazole has been recognized as a highly selective competitive inhibitor of P450 2C9 for some time [768] and has relatively poor affinity for other subfamily 2C P450 enzymes [671]. Mansuy's group examined some other similar compounds as ligands and inhibitors [764, 769]. Other inhibitors have been reported, although some have relatively poor affinity [770, 771], including several warfarin analogs [772]. For an early compilation of inhibitors, see Rendic [51]. Inhibitors of the subfamily 2C P450s have been reviewed more recently by Niwa and Yamazaki [660]. See also Table 9.6. Hanatani et al. [773] reported no differences in the effects of inhibitors on the *1 and *3 proteins (wild type and R144C), although it seems likely that some coding region variants may be found to differ.

Tienilic acid is a mechanism-based inactivator of P450 2C9 [774]. The mechanism involves *S*-oxygenation, and the unstable product reacts with P450 2C9 [775]. Subsequently, autoimmune antibodies develop in some patients who recognize unmodified P450 2C9 [774]. Exactly how (or if) this process is related to the hepatitis seen in some individuals who used tienilic acid is still unclear [776], but the phenomenon has raised concerns about whether such processes might be associated with other drugs that covalently modify proteins and could lead to idiosyncratic drug reaction in patients, one of the major concerns today for safety assessment in drug development. Structure–activity relationships have been reported on thiophenes other than tienilic acid [765].

A series of type II (spectra) π-binding ligands have been analyzed, in regard to their physical parameters [777]. Tienilic acid and (±) suprofen are mechanism-based inhibitors [778, 779].

Finally, some hydroxylated products of warfarin have been reported to be potent inhibitors of P450 2C9 [780], although not the ones derived from warfarin by P450 2C9.

9.7.9.7 Clinical Issues

One of the major current clinical issues regarding P450 2C9 is warfarin therapy (blood thinning for strokes). The safety margin is narrow, and too much warfarin can lead to internal hemorrhaging. There is a relationship between P450 2C9 genotype and warfarin dose [76, 781], and one issue is whether genotyping is useful in management of the drug [782]. Both negative [783] and affirmative [784–786] opinions have been expressed.

Another interesting issue regarding P450 2C9 involves the drug tienilic acid. The compound is a substrate and a mechanism-based inactivator of P450 2C9 [778]. A product of tienilic acid becomes selectively covalently bound to P450 2C9 (Sect. 7.9.6, *vide supra*). Some patients treated with tienilic acid develop liver injury (hepatitis). Some patients treated with tienilic acid also present with so-called liver–kidney microsomal (LKM) antibodies in their blood. These antibodies react with unmodified P450 2C9 [774]. Although it could be proposed that the modified P450 2C9 produces these autoantibodies and that they are involved in the liver injury, a causal relationship has never been demonstrated.

Genetic variations in P450 2C9 can lead to elevated levels of meloxicam [787] and celocoxib [788]. Polymorphisms have also been related to the response to celocoxib in cancer prevention [789].

The incidence of the *2 genotype has been related to bosentan-induced liver injury [790, 791]. The *2 genotype has also been reported to increase the risk for hypoglycemia in diabetic patients treated with sulfonylureas (e.g., tolbutamide) [792].

Finally, P450 2C9 genetic variation has been reported to contribute to the incidence of stroke [793] and to colorectal cancer [794].

9.7.10 P450 2C18

9.7.10.1 Sites of Expression

Relatively little has changed regarding P450 2C18 since the previous edition of this chapter was published [149]. Of the four human P450 subfamily 2C members, the level of hepatic expression is lowest for 2C18, at both the mRNA [629, 795, 796] and protein levels [297, 796, 797]. In intestine, P450 2C18 mRNA levels were high, but no protein was detected [796]. Expression in lung and skin has been reported to be significant [382, 797–800].

9.7.10.2 Regulation

Relatively limited information is available about regulation of P450 2C18. The levels of P450 2C18 mRNA in human liver and intestine were both reported to vary 25-fold [796]. At the protein level, expression in liver is reported to be very low (<2.5 pmol/mg protein) [55, 797].

Rae et al. [387] reported that P450 2C18 was not inducible by rifampicin in human hepatocytes, in contrast to P450s 2C8, 2C9, and 2C19.

In a humanized transgenic mouse model, P450 2C18 was expressed in liver and kidney in a male-specific manner [801], but the relevance to humans is unknown.

9.7.10.3 Genetic Variation

Variations in the *CYP2C18* gene have been reported [802] but are not included on the website http://www.cypalleles.ki.se. Effects on expression and catalytic activities are not well characterized. One variant has an exon 5 deletion [803].

9.7.10.4 Substrates and Reactions

P450 2C18 has low catalytic activity in tolbutamide methyl hydroxylation [803]. P450 2C18 is active in phenytoin metabolism, having an enzyme efficiency (k_{cat}/K_m) for 4-hydroxylation comparable to P450 2C9 and being more efficient in bioactivation to a reactive product [800]. Catalytic activities have also been reported with the substrates bisphenol A, diclofenac, the diclofenac derivative 2-[2(2,6-dichlorophylamino)] phenylethanol, and verapamil [660, 795]. Recently, P450 2C18 has been reported to oxidize 5-hydroxythalidomide to a reactive product (but does not catalyze the oxidation of thalidomide itself) [804].

9.7.10.5 Structure

No crystal structures have been published. Information about the active site of P450 2C18 is relatively limited beyond comparisons of the substrates mentioned above [795], the interaction of other P450 2C proteins with general P450 subfamily 2C substrates [659] and inhibitors [805], and inferences from the crystal structures of the other three P450 subfamily 2C crystal structures (i.e., 2C8, 2C9, 2C19). At least one homology model has been published [806].

9.7.10.6 Inhibitors

P450 2C18 is appreciably inhibited by sulfaphenazole, a classical inhibitor of P450 2C9. Mansuy's group has published on a set of sulfaphenazole derivatives that can be used in vitro [671, 672].

9.7.10.7 Clinical Issues

The limited expression and repertoire of catalytic activity for P450 2C18 still precludes consideration of any clinical issues at this time.

9.7.11 P450 2C19

Interest in P450 2C19 developed from the discovery of the polymorphic metabolism of the *S*-isomer of mephenytoin, the first major polymorphism to be studied following P450 2D6 [624, 625]. Initial work led to the purification of an enzyme with some (*S*)-mephenytoin 4′-hydroxylation activity [15]. Exactly how this and other gene products from the complex P450 2C subfamily [628, 689] were involved was unclear [807, 808]. Although there were some indications that the hexobarbital 3′-hydroxylase (P450 2C9) was the enzyme involved in mephenytoin hydroxylation [691, 809], expression of P450 2C9 cDNA [689] in yeast yielded a protein

with activity towards tolbutamide but not (*S*)-mephenytoin [627, 692]. P450 2C18 had also been suggested to be the enzyme [629].

Wrighton [630] compared (*S*)-mephenytoin 4′-hydroxylation activity in different liver samples with a protein gel band recognized by anti-rat P450 2B1 and correlated this with P450 2C19, a sequence which had been reported earlier. Subsequently, Goldstein et al. [22] expressed several subfamily 2C P450 cDNAs in yeast and identified P450 2C19 has having the highest activity with mephenytoin.

9.7.11.1 Sites of Expression

Apparently, significant expression only occurs in the liver. As with other human P450s examined to date, there appears to be no gender difference [810]. P450 2C19 has been detected in human liver microsomes using LC–MS proteomics methods [297]. P450 2C19 is a relatively minor P450 in its abundance, probably accounting for < 5 % of total P450 even in EM liver samples (Fig. 9.2) [54].

Neither P450 2C19 nor (*S*)-mephenytoin 4′-hydroxylation activity was detected in fetal liver samples [689].

9.7.11.2 Regulation

In vivo work had shown that the enzyme was inducible by rifampicin [811]. Thus, this P450 differed from P450 2D6 in that it was both polymorphic and inducible. Analysis of the regulatory system has not been extensive, but studies with human hepatocytes demonstrated induction of P450 2C19 mRNA by rifampicin, dexamethasone, and phenobarbital [698].

The regulation of transcription of P450 2C19 has been reviewed elsewhere [812]. P450 2C19 expression is downregulated by ER α [706]. Regulatory variations (e.g., *17) can increase rates of transcription (~twofold) [813], and this variant has been associated with peptic ulcer disease [814].

9.7.11.3 Genetic Variation

The variation and polymorphisms are now relatively well understood. The incidence of the PM phenotype in Caucasians is generally 2–3 %, but the incidence in Asians (at least Japanese, Koreans, Chinese) is ~20 % [167]. On some Pacific islands, the incidence has been reported to be as high as 75 % [815, 816]. In Thai, Burmese, and Karen populations, the incidence of PMs is "intermediate," i.e., 8–11 % [817].

The major defect in Caucasians and Japanese was first identified in an exon 5 mutation that leads to an aberrant splice site and yields a truncated protein [818]. Other variants are collected at the website http://www.ki.se/cypalleles/. These are rather diverse and include a mutation of the initiation codon [819] and altered enzymatic properties [815]. At the time of the update of this chapter, at least 48 allelic variants are known, with an additional 20 SNVs for which haplotypes have not been determined.

9.7.11.4 Substrates and Reactions

(*S*)-Mephenytoin 4′-hydroxylation is the classic reaction attributed to P450 2C19 (*vide supra*). Early studies on the basis of the polymorphism of tolbutamide hydroxylation suggested that the same enzyme might be responsible for both activities [626], but in vivo work [626] and heterologous expression studies [627] distinguished the two activities. Nevertheless, recombinant P450 2C19 has now been shown to have some tolbutamide hydroxylation activity [820].

A list of P450 2C19 reactions has been published by Rendic [51]. Another list of P450 2C19 substrates has been compiled, and catalytic efficiencies are compared to the other subfamily 2C P450s [660]. The scope of P450 2C19 in drug metabolism is rather significant (Fig. 9.1b, Tables 9.5, 9.6 and 9.7). One drug of particular interest is the ulcer drug omeprazole (and related compounds), because individuals with low enzyme activity show a better response to treatment for ulcers [79, 80]. Some of the early variations seen in warfarin metabolism [821] can be explained by the finding that P450 2C19 catalyzes the 8-hydroxylation of (*R*)-warfarin [822]. 18-Methoxycoronaridine is *O*-demethylated by P450 2C19 [823]. P450 2C19 is responsible for the 5- and 5′-hydroxylation of thalidomide, an older drug notorious for teratogenic effects that has been "rediscovered" [824]. Whether the ge-

netic variation is related to the birth defects is unclear.

P450 2C19 can also catalyze steroid oxidations, including progesterone 21-hydroxylation and testosterone 17-hydroxylations [825]. The organophosphate insecticide diazinon is activated in human liver by P450 2C19 [826].

One of the more well-studied substrates is the drug clopidogrel (Plavix®), which is converted to its active form in two steps, both catalyzed (in large part) by P450 2C19 [827] (see Sect. 7.11.7 regarding clinical issues). P450 2C19 is involved in the *N*-oxidation of voriconazole [828], and genotype is a major factor contributing to the highly variable in vivo pharmacokinetics [829]. Another substrate is the drug clobazam [830], and genetic variation in P450 2C19 affects the efficacy of therapy [831]. Other drug substrates of interest are escitalopram [832], fenbendazole [833], and thalidomide [834, 835].

As with other P450 2C subfamily enzymes, P450 2C19 activities are usually stimulated by cytochrome b_5 [736]. In this case, stimulation is not dependent on the heme in the cytochrome b_5 and thus electron transfer cannot be involved [736].

9.7.11.5 Structures

Johnson and his associates [749] have reported an X-ray crystal structure of P450 2C19 containing the inhibitor (2-methyl-1-benzofuran-3-yl)-(4-(hydroxy-3,5-dimethylpentyl) methanone. A comparison has been made with the available structures of P450 2C8 and 2C9 (*vide supra*). The size of the active site is similar to that of P450 2C9 and much smaller than that of P450 2C8.

Goldstein and her associates did chimera analysis and then site-directed mutagenesis on P450 2C9 to convert it to a protein with P450 2C19-characteristic omeprazole hydroxylation activity [836]. Only three changes were needed to achieve the activity of wild-type P450 2C19—I99H, S200P, and P221T. However, at least three different mutations were needed to convert P450 2C9 to an enzyme with (*S*)-mephenytoin 4′-hydroxylation activity, even to a catalytic efficiency one third of wild-type P450 2C19 (*1) [837]. In an opposite experiment, P450 2C19 was converted to a P450 2C9-like warfarin hydroxylase with high sensitivity to sulfaphenazole [766]. Residues 286 and 289 appear to be important. However, these residues may exert an indirect influence by adjusting the active site or substrate access channels [837].

9.7.11.6 Inhibitors

Niwa and Yamazaki [660] have compiled a list of inhibitors of subfamily 2C P450s. Two diagnostic inhibitors validated for P450 2C19 reaction phenotyping (in liver microsomes) are (+)*N*-3-benzylnirvanol [838] and (−)*N*-3-benzylphenobarbital [839]. The point has been made that the choice of "probe" substrates can influence in vitro inhibition profiles [840], which is not surprising in light of experience with P450 3A4 (*vide infra*). As indicated in Sect. 7.11.5 (*vide supra*), (2-methyl-1-benzofuran-3-yl)-(4-hydroxy-3,5-dimethyl)methanone was the inhibitor used to obtain the P450 2C19 crystal structure [749].

Two interesting inhibitors of practical interest are cannabidiol (marijuana constituent) [841] and grapefruit juice (extensively studied with P450 3A4) [842].

9.7.11.7 Clinical Issues

The issue is the genetic variation, particularly so for drugs marketed in Asian populations. At least eight alleles have been associated with the PM phenotype [816]. Desta et al. [816] reviewed some of the drugs for which the 2C19 phenotype is a problem (Tables 9.5, 9.6 and 9.7). Most pharmaceutical companies and regulatory agencies discourage development of a P450 2C19 substrate because of potential problems for PM individuals. Mephenytoin itself is seldom used and is not an issue. Several studies indicate that PM patients may have more effective therapy (for ulcers) with omeprazole and related compounds [816, 843–846]. The popular proton pump inhibitors omeprazole, lansoprazole, pantoprazole, and rabeprazole are metabolized by P450 2C19 (but not esomeprazole), and genetic variation is an issue in use for acid-related intestinal disease [847].

Another major drug of interest is clopidogrel (Plavix®), which is a P450 2C19 substrate

(converting the drug to the active form in two steps) [827]. The question has been raised as to whether the use of genotyping is useful in prescribing (correct doses of) this drug [848]. Both positive [849–852] and negative [853] opinions have been expressed. An Australian study concluded that genotyping for the use of clopidogrel was economically justified but for ticagrelor was not [854].

Other drug issues regarding P450 2C19 variation involve thalidomide therapy [835] and treatment of small-cell lung cancer with tivantinib and erlotinib [855].

As with many polymorphisms, epidemiology studies have been done to explore risks to diseases in the absence of information about etiology, substrates, etc. Some of the reports include suggestion of more hepatocellular cancer in PMs [856] and lack of association of leukemia with polymorphism [857]. Other possible relationships have been explored, but evidence for any associations is limited at this time [816]. Genetic variation in P450 2C19 has also been considered in regard to cancers of the breast (decreased with *17) [858], biliary tract [859], and digestive system [860]. Other diseases in which P450 2C19 genetic variation has been considered include endometriosis [861], essential tremor [862], peptic ulcers [814], and mortality following acute myocardial infarction [863].

9.7.12 P450 2D6

P450 2D6 is one of the main enzymes involved in drug metabolism (Fig. 9.1b). It was the first "xenobiotic-metabolizing" P450 recognized to be under monogenic regulation [11].

9.7.12.1 Sites of Expression

P450 2D6 is expressed mainly in liver and was first purified from liver microsomes [14, 19]. In the average person, P450 2D6 accounts for ~5% of total P450 (with wide variation) [52]. Estimates of the level of P450 2D6 vary in different studies. An older immunoblotting analysis of 60 samples (one-half Caucasian, one-half Japanese) showed a mean of 5 pmol P450 2D6/mg microsomal protein [52]. Similar levels were reported in adolescents by Stevens et al. [864]. One LC–MS analysis gave a mean of 30 pmol P450 2D6/mg microsomal protein [865], but a more recent LC–MS analysis gave a mean value of 12 pmol P450 2D6/mg microsomal protein [55]. Another yields values of 4–12 pmol P450 2D6/mg microsomal protein [54]. However, this enzyme is involved in the oxidation of ~25% of all drugs oxidized by P450s (Fig. 9.1b).

Developmental studies showed little P450 2D6 in early fetal liver and a rapid increase in protein shortly after birth, yielding a peak accumulation in newborns and decline in adulthood [866]. In another study, P450 2D6 levels increase during development, being low in fetal liver, increasing the third trimester and then somewhat high postnatally, increasing during childhood and adolescence [864].

P450 2D6 is also expressed at low levels in lung (bronchial mucosa and lung parenchyma) [867]. Another site of P450 2D6 expression is brain, with localization in large principal neurons [868]. Higher levels of brain expression have been reported in alcoholics [869].

In the central nervous system, there is evidence of several endogenous substrates and for neurophysiological differences in different genotypes (*vide infra*). Recently, a transgenic mouse line expressing human P450 2D6 has been developed and may provide insight [870].

P450 2D6 is generally considered a microsomal protein, but Avadhani and his associates have shown that an N-terminal chimeric signal in the protein (residues 23–33) also mediates targeting to mitochondria [871]. Naturally occurring variants can affect the localization, and phosphorylation has a role [872]. In the mitochondria, P450 2D6 is capable of using electrons from adrenodoxin, and the mitochondrial localization may be an issue in the bioactivation of the neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [873].

9.7.12.2 Regulation

All information available indicates that P450 2D6 is not inducible. Some factors are known to be involved in constitutive expression, including C/EBPα [547] and HNF4α [171].

Phosphorylation of P450 2D6 (in vivo) has also been reported [297].

9.7.12.3 Genetic Variation

The wide variability in the activity of P450 2D6 is attributed to genetic variation (Fig. 9.4). Reduced ability to metabolize the drug debrisoquine was first noted (personally) by Smith in a drug trial. Subsequent work led to the report of polymorphic hydroxylation of debrisoquine [11], including a phenotypic hypotensive response [874]. Racial differences were first noted in Africans [62]. The phenomenon of polymorphic debrisoquine hydroxylation [875] was also reported for sparteine oxidation [13, 876].

Today, P450 2D6 is considered to be a very polymorphic P450. At least 165 genetic variants are known (and 26 more not characterized for haplotype) (http://www.cypalleles.ki.se). The effects of formation of some have been identified [877] but not all (particularly the coding region variants, where function may vary depending upon the substrate and inhibitor). There is also variation of activity in vivo within each genotype [878], possibly due to differences in regulatory factors (or possibly the existence of endogenous or food-borne inhibitors).

The most significant decreases in activity for P450 2D6 alleles, aside from mRNA splicing problems and gene deletion [170], are considered to result from less stable proteins [879], although low-activity P450 2D6 variant proteins have also been reported [880, 881]. Some of the allelic differences are present as haplotypes [882].

In addition to the "poor" and "intermediate" metabolizer phenotypes, a "very extensive" or "ultra-metabolizer" (UM) phenotype was identified in early work (Fig. 9.4). Ingelman-Sundberg's group identified the basis for this as a gene duplication, with up to 13 copies present in some individuals [63]. The main form of this phenomenon is a haplotype resulting from gene duplication [63, 883]. The amplification appears to result from unequal segregation and extrachromosomal replication of the acentric DNA [884]. As many as 7% of Caucasians show some of this effect, and the incidence is even higher in some Middle Eastern populations [885].

9.7.12.4 Substrates and Reactions

Since the original work with debrisoquine [11], many substrates and reactions have been reported for P450 2D6. In some cases, the role of P450 2D6 is very dominant in vivo and the clinical manifestations of genetic polymorphism are important and even deadly [874, 886]. Lists of P450 2D6 substrates have been published [51]; see Table 9.5.

P450 2D6 catalyzes many of the basic kinds of oxidative reactions of P450s, e.g., aliphatic and aromatic hydroxylations, heteroatom dealkylations, etc. [887]. In early work in this laboratory [888], the observation was made that many of the substrates contained a basic nitrogen atom situated ~5 Å away from the site of oxidation, possibly due to a specific anionic charge in P450 2D6. Subsequently more detailed pharmacophore models have been developed [889–892]. All of these are based on the premise that a basic nitrogen atom in the molecule interacts (coulombic bond) with an acidic amino acid in P450 2D6, usually Asp-301 in most studies. (More recent work shows a role for Glu-216, however, *vide infra*.)

The use of these models requires some caveats. Although the pK_a of the substrate has been proposed to have a dominant influence [893], work in this laboratory has shown that the intrinsic pK_a of a substrate can be altered in the active site of P450 2D6 [894]. Another issue is that some compounds with a single amine nitrogen undergo *N*-dealkylation, e.g., deprenyl [895], which cannot be easily rationalized with an amine-oxidation site interatomic distance of 5–7 Å. Some substrates devoid of basic nitrogen (and any nitrogen) have been reported, including steroids [896, 897]. Spirosulfonamide and several analogs do not have a basic nitrogen but have been shown to be good substrates and ligands for P450 2D6 [898] (Fig. 9.16).

A large fraction of the population is devoid of functional P450 2D6 but appears to function well. This information may be interpreted to mean that P450 2D6 has no "physiological" substrate. Nevertheless, some reactions may be catalyzed by P450 2D6 and yield physiological responses that yield less than obvious changes. For instance,

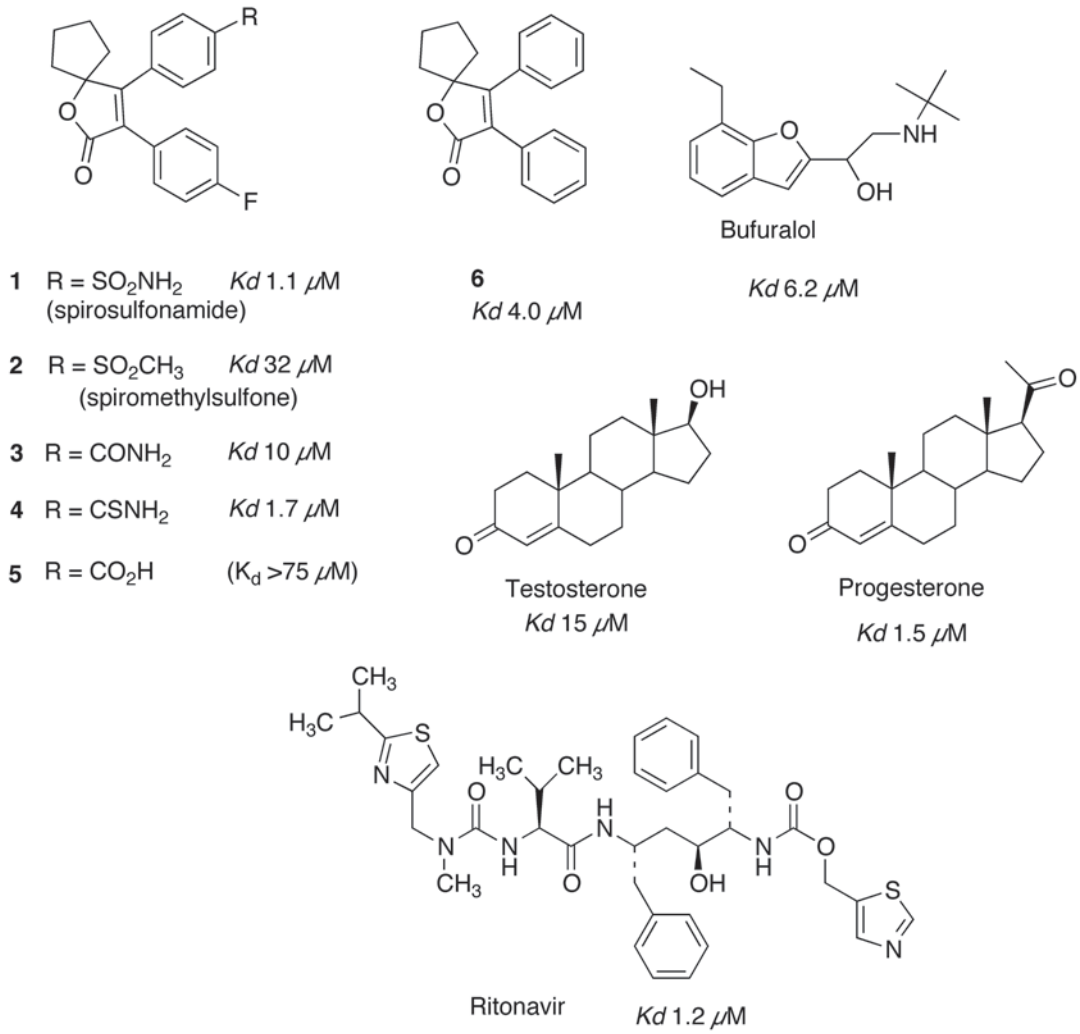

Fig. 9.16 Analogs of spirosulfonamide and other P450 2D6 ligands. K_d values were estimated by spectral titration [898]. (With kind permission from Springer Science + Business Media: [149], Fig. 10.10)

overexpression of human P450 2D6 in transgenic mice produces a higher capability to adapt to anxiety [870]. Tryptamine has been proposed as a physiological substrate in one study [899] but discounted in another [900]. Proposed physiological reactions catalyzed by P450 2D6 are the *O*-demethylation of 5-methoxytryptamine, 5-methoxy-*N*,*N*-dimethyltryptamine, and pinoline (6-methoxy-1,2,3,4-tetrahydro-*β*-carboline) [900, 901]. Whether significant catalysis is seen at the low concentrations seen in vivo and what the effect is remains to be established.

P450 2D6 catalyzes tamoxifen *α*-hydroxylation and formation of *α*,4-dihydroxy tamoxifen [902]. P450 2D6 has been reported to be the major enzyme involved in the *O*-demethylation of the designer drug *p*-methoxymethamphetamine [903]. MPTP, a breakdown product of a designer drug, is oxidized by P450 2D6-catalyzed aromatic hydroxylation and *N*-demethylation [904]. P450 2D6 can also convert MPTP to MPP^+ (1-methyl-4-phenylpyridine), as shown in mitochondria, and contributes to neurotoxicity in the substantia nigra [873].

Possible endogenous substrates have also been considered, including 5-methoxyindolethylamine [900]. Human P450 2D6 also catalyzes some important steps in mammalian opioid biosynthesis, including conversion of (*R*)-reticuline to salutaridine, thebaine to oripavine, and codeine to morphine (Fig. 9.17) [905, 906].

Modi et al. [907] reported differences in product profiles of P450 2D6 reactions supported with artificial oxygen surrogates and NADPH-P450 reductase and interpreted these as evidence for an allosteric influence of the reductase. Subsequent experiments in this laboratory did not support this conclusion and are in accord with some differences in the chemical mechanisms for the oxygen surrogates [908].

Detailed experiments have been done on the *O*-demethylation of 3- and 4-methoxyphenethylamine by P450 2D6 [909]. Analysis of kinetic deuterium isotope effects, kinetic simulation, and other experiments yields evidence that both late steps in O_2 activation and C–H bond breaking contribute to k_{cat}. The exact meaning of K_m is still not defined with this and most P450 reactions. Some of the P450 2D6 allelic variants show no changes in k_{cat} for certain reactions but do show K_m differences [910]; these are probably more complex than simple "affinity" for the substrate.

9.7.12.5 Structure

The active site of P450 2D6 has been the subject of considerable interest, probably because of the relevance to issues in the pharmaceutical industry. Some residues have been identified as being important, and early homology and pharmacophore models have been published [889–892, 911–917].

The original clone reported by Gonzalez [34] had Met at position 374, but this now appears to be a gene variant or artifact; the correct residue is Val [918, 919]. This residue appears to be in the active site and affects activity.

Rowland et al. [920] published an X-ray crystal structure of a slightly modified P450 2D6 without a ligand. Johnson and his associates [323] subsequently published a structure of P450 2D6 with the ligand prinomastat bound. The latter structure had the *1 Val-374 instead of Met-374 and differed significantly from the earlier P450 2D6 structure devoid of a ligand. The differences in the structure are attributed to the flexibility of P450 2D6 and conformational changes seen with binding [323]. High-pressure experiments indicate that P450 2D6 is a much more rigid molecule when a substrate is bound [921].

The crystal structures indicated that both Asp-301 and Glu-216 are in position to form ionic bonds to charged amines [323]. Although much of the earlier literature was focused on Asp-301, both Asp-301 and Glu-216 have anionic changes that are used in binding positively charged substrates [922, 923]. Interestingly, site-directed mutagenesis of a few residues of P450 2D6 allowed oxidation of quinidine [924], which is only an inhibitor of the wild-type enzyme [97]. Previous studies had shown that neutral molecules are ligands of P450 2D6 (Fig. 9.17) [898], in contrast to earlier views about the need for basic atoms in ligands. Even acidic (e.g., pactimibe) molecules can be ligands and substrates [925].

Newer predictive pharmacophore schemes have been developed, some based in part on the available crystal structure of P450 2D6 [926–928].

9.7.12.6 Inhibitors

Many inhibitors of P450 2D6 have been reported (Table 9.6) [51, 890]. Inhibition of P450 2D6 is an undesirable issue in drug development, and most pharmaceutical companies have screening programs in place. As with some other P450s (e.g., P450 3A4, *vide infra*), inhibitor screening results have been reported to be dependent upon the test substrate used [929]. Structure–activity relationship studies have been done with quinidine analogs [930].

The most established inhibitor of P450 2D6 is quinidine [931]. The K_I is ~50 nM and inhibition is competitive. Interestingly, quinidine is not a substrate for P450 2D6 [97, 909].

Mechanism-based inactivation of P450 2D6 is known, e.g., 5-fluoro-2-[4-[(2-phenyl-1*H*-imidazoyl-5-yl)methyl]-1-piperazinyl]pyrimidine (SCH66712) [932]. In the case of this compound, covalent binding to protein was detected, but the position of attachment has not been identified.

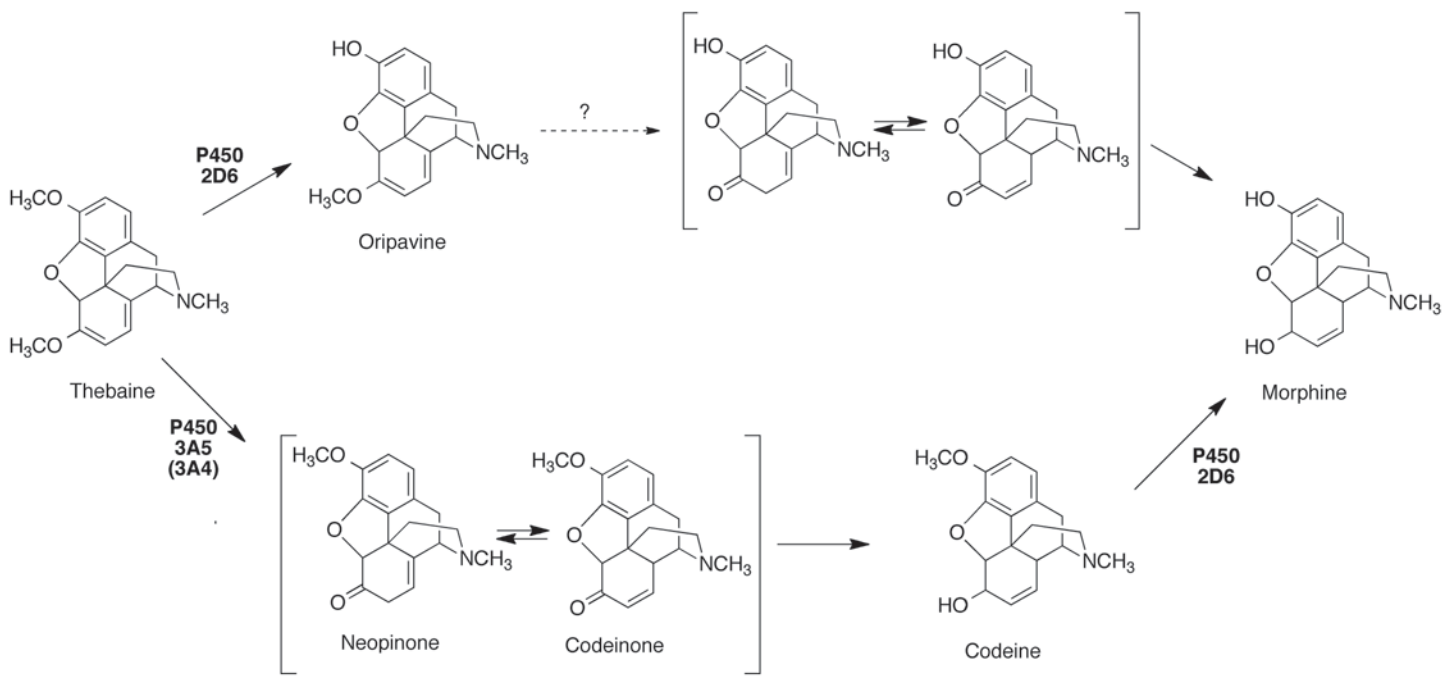

Fig. 9.17 Steps in mammalian morphine synthesis [905, 906]. P450 3A4 and 3A5 catalyze the O^6-demethylation of thebaine with 3A5 being ~10-fold more active than P450 3A4 [906]. The microsomal oxidation of oripavine is very slow and is considered not to contribute. The conversion of codeinone to codeine is catalyzed by a reductase. (Adapted with kind permission from Springer Science + Business Media: [149])

Two related mechanism-based inactivators, 1-[2-ethyl-4-methyl-1*H*-imidazol-5-yl)methyl]-4-[4-(trifluoromethyl)-2-pyrinyl]piperazine [933] and 5-fluoro-2-[4-[(2-phenyl-1H-imidzo-5-yl)methyl]-1–1piperazinyl]pyrimidine [934], modify the apoprotein. Several other mechanism-based inactivators of P450 2D6 have been reported [935], including methylenedioxymethamphetamine [936] but not metoclopramide [937].

Other reported P450 2D6 inhibitors include sanguinarine [938] and cannabidiol, a marijuana constituent [939].

9.7.12.7 Clinical Issues

The clinical issues regarding P450 2D6 are considerable due to the large variation in the genetics in the population (Figs. 9.1b, 9.5, and 9.6) and the contribution of P450 2D6 in the total scheme of drug metabolism (Fig. 9.1b). Individuals seem to be rather tolerant of the wide variability in expression with many marketed drugs, probably because of generally wide therapeutic windows selected for in the basic process of drug development. However, P450 2D6 PMs can be at considerable risk when they encounter certain drugs, as first observed by Smith [11, 874]. The problem is seen with drugs having a relatively narrow therapeutic index, e.g., debrisoquine [11], phenformin [940], captopril [941]. The effects of P450 2D6 deficiency are seen not only in short-term treatments but also in long-term therapy [942]. The issue of ineffectiveness of drugs that are very rapidly metabolized by "very extensive" (UM) metabolizers is an issue (Fig. 9.4). Modeling of the variability is still an issue [943] and may be a function of particular drugs. The issue of whether genotyping/phenotyping is economical has been considered, particularly in the case of neuroactive and antipsychotic drugs [944, 945]. The overlap between P450 2D6 substrates and neuroactive drugs is also an issue in drug development, largely due to the overlap of these two groups of compounds [946].

Zhang et al. [947] have commented on the role of genetic polymorphisms in withdrawal of drugs from the market. Another concern, in the context of drug–drug interaction, is "phenoconversion," making an individual a "PM" due to inhibition (e.g., prescribing quinidine to a P450 2D6 "EM") [948].

Although it seems very possible that individuals might die due to drug interactions related to genetic variations, there is actually only very limited evidence that this has happened. A study of individuals who died due to drug toxicity did not show any relationships to known genetic variations in P450s [949]. There is a report of near-fatal tramadol cardiotoxicity in a P450 2D6 ultrarapid metabolizer [950]. In 2004, an infant died due to codeine intoxication when nursing from his mother, who was an ultrarapid (UM) P450 2D6 metabolizer (and generated an overdose of morphine) [81].

One of the substrates of P450 2D6 is tamoxifen [902], an ER antagonist used extensively in breast cancer therapy. There has been controversy regarding application of genotyping to improve therapy. Recommendations in favor of genotyping and against it [951] have appeared, and several meta-analyses conclude that more study is needed [952–954].

Another issue with P450 2D6 is the relevance of the genetic variation to cancer risks. In 1984, Idle [123] reported an association of lower risk of lung cancer (in smokers) with the P450 2D6 PM phenotype. These epidemiological results were repeated in some studies [955] but not others [124]. Attempts were made to resolve the discrepancies on the basis of levels of smoking [956]. Although some expression of P450 2D6 is detectable in lung [867], no clear role for P450 2D6 in carcinogen activation could be established, even with crude tobacco smoke fractions [125]. The issue of whether lung cancer is associated with P450 2D6 was not resolved by changing analyses from phenotyping to genotyping. The generally accepted epidemiological conclusion today is that P450 2D6 is not related to lung cancer [124, 957–960].

Other epidemiology studies have suggested relationships of P450 2D6 with other cancers [961, 962], but these findings have not been scrutinized as rigorously as the lung cancer hypothesis.

Another disease in which P450 2D6 has been proposed to play a role, on the basis of epidemiol-

ogy, is Parkinson's disease [963]. Contradictory findings have been reported [964, 965]. Although a hypothesis has been raised that induction of P450 2D6 by smoking might explain some discrepancies [966], this proposal lacks biological plausibility in light of the known refractory response of P450 2D6 to induction. Some positive evidence for risk of Parkinson's disease with "PM" P450 2D6 status has been published [953].

Autoantigens (LKM1) that recognize P450 2D6 have been known for some time [967, 968]. These antibodies are associated with some cases of hepatitis. The exact mechanism of how they arise is still unclear, as is the relationship with hepatitis. The antibodies may arise by molecular mimicry [969] or they may result from P450 2D6 translocation to the outer plasma membrane [970, 971]. These LKM1 antibodies may serve as diagnostic tools for particular types of hepatitis [972, 973], but causal relationships have never been demonstrated.

P450 2D6 genetic variation has been considered, with some evidence, in explaining depression [974], suicide in relation to serotonin use [975], and type A versus type B personality [976].

9.7.13 P450 2E1

The microsomal mixed-function oxidation of ethanol was reported nearly 50 years ago [977]. The view that ethanol could be a P450 substrate was not readily accepted because of the hydrophilic nature of the molecule, but Lieber's group characterized the enzymatic reaction in rat liver [978, 979]. Collaborative work with Levin led to the isolation of a P450 ("j"), which was also found to be inducible by isoniazid [980]. Human P450 2E1 was purified by Wrighton et al. [23], and Gonzalez's group characterized the human gene [981].

9.7.13.1 Sites of Expression

The greatest concentration is in the liver, and P450 2E1 is a moderately abundant P450 (Fig. 9.2). Using LC–MS proteomics, Shrivas et al. [635] detected P450 2E1 in all human liver microsomal samples analyzed. Seibert et al. [982] used LC–MS and reported values of 88–200 pmol (P450 2E1)/mg microsomal protein in four samples, which seems unusually high compared to other P450s (Fig. 9.2) [55] and an average total of ~500 pmol/mg protein [10]. The interindividual variation is an order of magnitude (Fig. 9.6) [52, 983]. A racial difference exists, with Japanese samples having mean expression levels lower than Caucasians (Fig. 9.6) [69].

P450 2E1 was reported not to be present in fetal liver but appears within a few hours after birth, regardless of the gestational age [984]. However, P450 2E1 has also been reported to be detectable as early as gestational day 93 in fetal liver [985]. The activity increases during the first year of childhood, and transcriptional regulation due to hypermethylation has been proposed.

P450 2E1 is expressed in many extrahepatic sites, including lung [535], esophagus, small intestine [382], brain [986, 987], nasal mucosa [988], and pancreas [989] (some of the evidence is extrapolated from rat work and not necessarily extendable to humans).

P450 2E1 is found mainly in the endoplasmic reticulum. With heterologous expression in bacteria (rabbit), P450 2E1 was membrane bound and catalytically active even when amino acids 3–29 are deleted [38, 990]. The same bacterial localization was seen with human P450 2E1 from which 21 N-terminal residues were deleted [991]. However, P450 2E1 can show some unusual localization in mammalian systems. Ingelmann-Sundberg's group deleted residues 2–29 of rat P450 2E1 and demonstrated the presence of a fragment in the mitochondria of a mouse hepatoma cell line [992]. Avadhani's group found P450 2E1 intact in rat liver mitochondria and reported that it could couple with adrenodoxin and adrenodoxin reductase with full catalytic activity [993]. Subsequent work demonstrated a cryptic mitochondrial-targeting signal at positions 21–31 that was activated by cyclic AMP-dependent phosphorylation of Ser-129 [994]. Neve et al. [995] found that the charge of the N terminus of (rat) P450 2E1 was such that part is directed to either the lumen of the endoplasmic reticulum or the outside of the plasma membrane. Migration of human P450 2E1 into mitochondria and the

relevance to oxidative stress have been published [996, 997].

9.7.13.2 Regulation

Early work in experimental animals was focused on the induction of P450 2E1 in rat liver [978]. Subsequently many other chemicals, including isoniazid and some solvents, were shown to induce P450 2E1 [998]. It is also of interest to note that some of the common polycyclic hydrocarbons and other inducers of P450 1 family enzymes attenuated the level of P450 2E1 in rats [998]. The regulation of P450 2E1 has come to be recognized to be relatively complex, involving transcriptional activation, mRNA stabilization, increased mRNA translation efficiency, and decreased protein degradation [999].

HNF-1 is believed to regulate *CYP2E1* gene transcription [183]. Obesity and diabetes are known to modulate P450 2E1 in rat models. In rat hepatocyte cell culture, insulin attenuated mRNA levels and glucagon or dibutyryl cyclic AMP elevated mRNA, with the latter effect down-regulated by a protein kinase A inhibitor [1000]. mRNA levels are also selectively attenuated in mice or cell culture (relative to other P450s) by interleukin-6 [1001], interleukin-4 [1002], or interleukin-1β or tumor necrosis factor (TNF)α [1003]. Multiple mechanisms have been invoked, including kinase pathways, control of HNF-1α function, and regulation of other transcription factors.

Evidence for control at the level of mRNA stability and enhanced translation efficiency has been presented by Novak [182, 1004]. The 3′-region of the gene appears to be important in stability. The relevance of this rat model to human P450 2E1 is still unknown.

Another mechanism, generally well accepted although not completely understood, involves protein stabilization by substrate. Rat studies (in vivo) showed that ~half of P450 2E1 was lost in 1 h, and a ubiquitin-linked pathway was invoked [1005]. Similar findings were also reported for human P450 2E1 in HepG2 cells [1006]. An attempt has been made to estimate the half-life of P450 2E1 in humans in vivo using chlorzoxazone pharmacokinetics and a P450 2E1 inhibitor [1007]. The half-life was estimated at 50 ± 19 h, but this approach may not be sensitive enough to detect a short-lived P450 2E1 pool.

Some aspects of P450 2E1 regulation have been reviewed by Gonzalez [184]. P450 2E1 is also regulated by miR-378 [1008]. Daly has reviewed genetic variation involving P450 2E1 gene regulation [1009].

P450 2E1 phosphorylation has been detected in vivo [297], although the relevance is not yet clear.

9.7.13.3 Genetic Variation

P450 2E1 is polymorphic. At least 14 allelic variants of P450 2E1 are known, with four additional variants for which the haplotype has not been determined (http://www.cypalleles.ki.se). In some cases, the functional effects of coding region substitutions have been defined [1010]. Because of the nature of many of the substrates, many efforts have been made to determine the relevance of SNPs and other variations to disease and risk of injury. A polymorphism in the 5′-flanking region was suggested to be related to the binding of a transcription factor and related to alcohol intake [168, 1011]. A number of other polymorphisms have been identified [168, 1012, 1013]. However, the evidence to date indicates that these variations do not seem to have much significance in terms of their effects on in vitro or in vivo activity of P450 2E1 [69, 1012, 1014–1016].

9.7.13.4 Substrates and Reactions

P450 2E1 was originally characterized as an ethanol-oxidizing enzyme. P450 2E1 can oxidize some compounds that are present in the body, including acetone and possibly other ketones involved in certain physiological syndromes (fasting, diabetes) [1017]. Transgenic P450 2E1-knockout mice appear to be relatively normal, although the blood acetone levels become much higher (than in wild-type mice) after fasting [1018].

The role that P450 2E1 plays in ethanol metabolism has been debated for many years [1019]. What seems to be the general consensus is that alcohol dehydrogenase is the main enzyme involved in ethanol oxidation. The overall contribution of P450s to the oxidation of etha-

nol is considered elsewhere, relative to alcohol dehydrogenase and catalase [1020]. The point is made that even if an overall role of an enzyme (P450) is low, there may be strong "local" effects. Somewhat surprisingly, the experimental survey of human P450 enzymes did not show a strong role for P450 2E1 relative to other P450s [1021]. P450 2E1 may contribute at very high ethanol concentrations or in individuals with low levels of alcohol dehydrogenase activity. P450 2E1-knockout mice have blood ethanol levels not significantly different from wild-type animals after administration of ethanol [1022]. Acetaldehyde, the product of ethanol oxidation, is also oxidized to acetic acid by rat and human P450 2E1 (Fig. 9.19) [1023–1025].

The oxidation of 4-nitrophenol to 4-nitrocatechol has been used as an in vitro marker of human P450 2E1 [1026]. Chlorzoxazone 6-hydroxylation was demonstrated to be a relatively specific reaction catalyzed by human P450 2E1; other enzymes (e.g., P450 1A1) can catalyze the reaction but with poor catalytic efficiency [1027, 1028]. Chlorzoxazone is a relatively innocuous muscle relaxant, and the assay can be used in vivo to estimate hepatic P450 2E1 function noninvasively [69, 1016].

One group of substrates of interest is *N*-alkylnitrosamines, which are carcinogens at many sites and can be formed by chemical reactions within the body (e.g., stomach acid) [1029]. Early research on the activation of *N*-nitrosodimethylamine (*N,N*-dimethylnitrosamine) indicated biphasic kinetics of the activating *N*-demethylation reaction in liver microsomes and the possible contribution of multiple P450s and possibly other enzymes [1030, 1031]. The enzyme involved in the "low K_m" reaction was shown to be P450 2E1 in rat and human liver [1032, 1033]. An in vivo role of P450 2E1 has been confirmed in rats [1034]. However, P450 2A6 has a significant share of the role of activation of some more complex nitrosamines, even *N*-nitrosodiethylamine [432, 433]. The oxidation of *N*-nitrosodimethylamine is actually a two-step reaction leading to formic acid, which appears to be relatively processive (Fig. 9.18) [1035].

P450 2E1 has been shown to be a major P450 involved in the oxidation of a number of low molecular weight procarcinogens, including not only nitrosamines but also benzene, styrene, CCl_4, $CHCl_3$, CH_2Cl_2, CH_3Cl, CH_3CCl_3, 1,2-dichloropropane, ethylene dichloride, ethylene dibromide, vinyl chloride, vinyl bromide, acrylonitrile, vinyl carbamate, ethyl carbamate, and trichloroethylene [461]. The oxidations by P450 2E1 all have relevance to the activation and detoxication of these compounds and their risk assessment (Figs. 9.9 and 9.10) [461, 1036]. Another substrate is the gasoline additive methyl *tert*-butyl ether [1037]. A role of P450 2E1 has been shown in the activation of some of these chemicals in knockout mice [1038, 1039].

Another substrate for human P450 2E1 is lauric acid, which undergoes 11-hydroxylation [1040, 1041]. The physiological relevance of this reaction is unknown. Indole is oxidized by P450 2E1 (3-hydroxylation to indoxyl, generating indigo) as well as by other P450s, particularly P450 2A6 and 2C19 [446, 1042]. The relevance of this reaction to the urinary excretion of indigoids [1043] is still unclear.

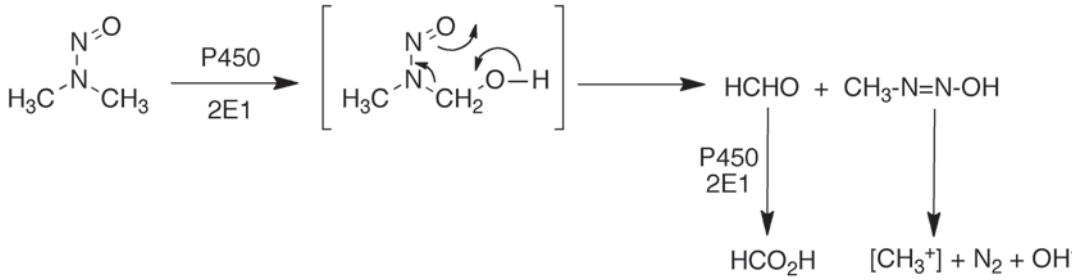

Fig. 9.18 Sequential oxidation of *N,N*-dimethylnitrosamine to formaldehyde and formic acid by P450 2E1 [1035]

Relatively few drugs are oxidized by P450 2E1 (Fig. 9.1b). Chlorzoxazone is one [1027]. Halogenated anesthetics are often metabolized by P450 2E1, including halothane [1044] and isoflurane [1045]. For more substrates, see Rendic [51].

Another example of an *N*-oxygenation by P450 2E1 has been reported, that of nicotinamide [1046], to go with pyridine *N*-oxygenation.

A detailed kinetic analysis of the human P450 2E1-catalyzed oxidation of ethanol showed that the product acetaldehyde was converted to acetic acid in a rather processive manner [1025, 1047]. Both reactions occur with burst kinetics, i.e., a rate-limiting step occurs after product formation, and the actual rate of oxidation (formal C–H bond cleavage) is very fast [1047]. Similar phenomena were observed with P450 2E1 oxidation of *N*-nitrosomethylamine (*N,N*-dimethylnitrosamine), in terms of oxidation of the resulting formaldehyde to formic acid (and processive oxidation of *N*-nitrosodiethylamine to acetaldehyde to acetic acid) [1035]. This processivity is rather unique to P450 reactions, including steroid hydroxylations [220] but has also been observed with P450 2A6 in nitrosamine oxidations [1048]. These phenomena are related to the expression of kinetic deuterium isotope effects in the K_m parameter [1025, 1047]. The intermolecular isotope effect is expressed in the K_m parameter, which includes the C–H bond-breaking step. k_{cat} is governed largely by an enzyme physical step after oxidation of the substrate. In this system, the K_m term contains k_{cat} as a variable [1025, 1047, 1049]. The reasons for the processivity in these reactions are not clear yet, in that there does not appear to be an intrinsic chemical affinity for the aldehyde products to P450 2E1 (or P450 2A6) [1025, 1047, 1048, 1050]. One possibility, which can be rationalized in kinetic models, is that a conformational change occurs after the initial substrate binding and that this stays "locked" after the aldehyde forms, leading to a favorable oxidation of the aldehyde [1048].

One of the issues in P450 2E1 in vitro reactions is the need for cytochrome b_5, first demonstrated with the rat enzyme [1032] and also the human enzyme [1033, 1047]; the involvement also exists in microsomes [1051]. Cytochrome b_5 also augments P450 2E1 activity in bacterial expression systems [736, 1052]. In contrast to several of the P450s, apo-cytochrome b_5 (minus heme) does not function, arguing for a "classic" role of electron donation in enhancement of catalysis [736, 1053].

Other unusual phenomena have been reported in P450 2E1 reactions, including negative cooperativity and inhibition at high substrate concentrations [1054]. These effects have been rationalized in terms of multiplicity of ligand binding, although there has been no structural support for this hypothesis yet (Sect. 7.13.5, *vide infra*).

Mathematical models have also been developed for rates of oxidation by P450 2E1 [1055, 1056]. In essence, these are based on chemical reactivity at individual substrate atom sites. In both of the cited examples [1055, 1056], the models were used for relatively small sets of related compounds and may have some utility. An inherent problem in more extended sets is the difficulty in interpretation of the parameters k_{cat} and K_m. Thus, the rate-limiting step may not be related to hydrogen abstraction or a similar chemical step involving the substrate (*vide supra*).

9.7.13.5 Structure

In 2008, Scott and her associates reported X-ray crystal structures of human P450 2E1 with imidazole and the inhibitor 4-methylpyrazole bound [1057]. Her group has also published structures with imidazole-modified fatty acids [1058] and pilocarpine [456]. The structures reveal an extra pocket near the binding site of a small molecule, and with different ligands the size of the active space available to the substrate can vary from 190 to 470 Å^3 [456]. Thus, P450 2E1 is somewhat flexible, and this behavior can explain the range in the size of substrates from ethanol to long-chain fatty acids (Sect. 7.13.4, *vide supra*).

A pharmacophore template for prediction of oxidations by P450 2E1 has been published by Yamazoe et al. [1059].

The kinetics of CO binding to human P450 2E1 following flash photolysis [1060] appeared to be monophasic and the rate was decreased in the presence of (400 mM) ethanol. One interpre-

tation of the results is that binding of the substrate makes P450 2E1 more rigid [1060].

9.7.13.6 Inhibitors

As mentioned earlier, many low molecular weight solvents are substrates for P450 2E1. These are also inhibitors of P450 2E1 [155, 156]. Such inhibition is a problem in that historically many insoluble P450 substrates are added to enzymes with solvent concentrations of 1% (v/v), which is often ~100 mM, and thus care is needed in analyses. It is possible to dilute many of the P450 2E1 low molecular weight substrates directly in water to add them to incubations, e.g., methylene chloride (normally considered immiscible) has a solubility of ~100 mM in H_2O [1061].

Some of the alcohol and aldehyde dehydrogenase inhibitors are also inhibitors of P450 2E1, making interpretations of in vivo ethanol metabolism studies difficult. 4-Methylpyrazole is an excellent inhibitor [314, 1062] and probably the best one for in vitro experiments at this time. 3-Amino-1,2,4-triazole [1063] and diethyldithiocarbamate [461] are mechanism-based inactivators. The latter is of interest in that the oxidized form, disulfiram (Antabuse®), is an aldehyde dehydrogenase inhibitor used with patients in alcohol aversion therapy. Many of the early animal and human studies on interactions of ethanol and disulfiram with various chemicals can now be rationalized in the context of P450 2E1 [1064, 1065].

A number of compounds of natural origin have also been examined as P450 2E1 inhibitors, many of which are derived from vegetables such as onions, garlic, and cruciferous vegetables [1066, 1067].

In addition, the characterization of mechanism-based inhibition of P450 2E1 by diethyldithiocarbamate [1068], 3-hydroxyacetanilide (the "*meta*" isomer of acetaminophen) [1069], and the chemopreventive agent phenethyl isothiocyanate [1070] have been reported. The inhibition by diethyldithiocarbamate has been proposed to involve modification of one of the thiol groups of P450 2E1 [1068].

9.7.13.7 Clinical Issues

Gonzalez has reviewed some of the clinical and practical aspects of P450 2E1 [184], which include the role of P450 2E1 in the oxidation of certain drugs, alcoholism, oxidative stress, and risk from cancer.

As pointed out earlier, the most generally accepted noninvasive human assay involves 6-hydroxylation of the muscle relaxant chlorzoxazone [1016, 1027]. Studies with humans show little effect of diabetes [1016, 1071] but an effect of body weight/obesity [1071, 1072]. As mentioned before, genotype has shown little impact on the in vivo parameters to date [69, 1072].

Another issue is drug metabolism and toxicity. Acetaminophen (paracetamol) overdose remains a major cause of liver failure in the USA and Europe. Several P450s are involved in the oxidation to the reactive iminoquinone [304]. Studies with P450 2E1-knockout mice indicate that P450 2E1 is probably a major determinant of acetaminophen toxicity in humans, because the toxicity was considerably attenuated in P450 2E1-knockout animals [104].

P450 2E1-null mice have the same blood ethanol levels as wild-type animals after ethanol dosing [1022], suggesting that P450 2E1 activity is not a major factor in ethanol metabolism, at least in mice. The situation regarding a role for P450 2E1 in alcohol-induced liver injury in other models is unclear, with some reports suggesting a link [1073, 1074] and others not [1022, 1075]. Autoantibodies against P450 2E1 have been reported in alcoholics [1076] and attributed to hydroxyethyl radicals [1077] (which may arise from lipid peroxidation processes rather than as intermediates in P450-catalyzed oxidation, *vide supra*). P450 2E1 is also a major autoantigen associated with halothane hepatitis, a rather idiosyncratic response [1078]. As with other autoimmunities involving P450s (2C9, 2D6, 21A2, *vide supra* and *vide infra*), causal associations remain to be demonstrated.

Many studies have been reported on the relationship of *CYP2E1* genetic variations to risk of diseases. Benzene poisoning in Chinese workers

showed some changes in risk with one genotype but only in smokers. With regard to cancers, the results appear to be very mixed. An early report suggested a link of lung cancer with a polymorphism, but since then the results have been mixed for cancers of the lung [1079–1084], oral cavity [1085, 1086], and stomach [1087]. In most of these cases, it should be emphasized that there is little information about exposure and the only relevant etiology is probably tobacco-derived nitrosamines. In a study of workers exposed to vinyl chloride (a P450 2E1 substrate [461]), some association was found between a P450 2E1 polymorphism and p53 mutations [1088]. However, it should be emphasized again that the relevance of *CYP2E1* genetic variants to known P450 2E1 reactions is unclear, particularly in vivo [1072], and it is difficult to define roles of these genetic polymorphisms in cancer risk; overall, P450 2E1 expression due to environmental influences may have a role but is more difficult to establish.

Because of the role of P450 2E1 in the metabolism of industrial chemicals, there is considerable interest in the field of occupational medicine [1089]. Genetic variations of P450 2E1 in human population have been linked to vinyl chloride-induced liver fibrosis [1090] and risk assessment of volatile organic chemicals [1091]. Physiologically based pharmacokinetic models have been developed to incorporate variation in human population, using trichloroethylene as an example [1092]. Efforts have been made to relate genetic variations in P450 2E1 to cancer of the lung [1093], head and neck [1094], gastric tract [1095, 1096], and colon/rectum [1097] and various chemically induced cancers [1098].

Autoantibodies to P450 2E1 have also been detected in cases of chronic hepatitis C infection [1099, 1100].

There is an extensive literature relating P450 2E1 to generation of reactive oxygen species and oxidative stress, e.g., [1101–1104]. Ingelman-Sundberg reported that P450 2E1 contributed ~20% of the NADPH-dependent lipid peroxidation in rat liver microsomes (and 45% in microsomes prepared from rats treated with acetone to induce P450 2E1) [1105]. Transfection of human P450 2E1 into a rat hepatic stellate cell culture system elevated the production of reactive species [1106]. Cederbaum [1107] has reviewed studies on the relationship of oxidative stress to P450 in liver cell models. However, almost all of the studies on P450 oxidative stress are in vitro studies (including cell culture), and there have been few in vivo studies. Even in the in vivo work that has been done, the biomarkers for oxidative stress are not ideal [1108, 1109]. Results from this laboratory showed that F_2 isoprostanes, considered the most reliable biomarkers of oxidative stress [1110], were not altered in rats treated with isoniazid to induce P450 2E1 [1111]. The same findings were observed (for liver, kidney, brain, and urinary isoprostanes) in mice [1112]. Further, no differences in the levels of the isoprostanes were seen between *CYP2E1*$^{+/+}$ and *CYP2E1*$^{-/-}$ mice. Mice with an Nrf2 reporter transgene system did not show increased activity when treated with isoniazid to induce P450 2E1 and did not show changes [1112], in marked contrast to in vitro studies on P450 2E1 in HepG2 cell culture [1102]. Although "global" oxidative stress does not appear to be associated with P450 2E1 in rodent models, the production of local "pockets" of reactive oxygen species, e.g., in mitochondria (as documented by isoprostane formation in in vitro systems [997]).

P450 2E1 may also be involved in nonalcoholic fatty liver disease, although this area is also controversial and genetic variations have not been implicated [184, 1113].

9.7.14 P450 2F1

9.7.14.1 Sites of Expression

P450 2F1 was originally cloned from a human lung library [1114]. It is expressed in bronchial epithelial cells. This is considered a lung-specific P450, although there have been some repeats of protein expression in liver [635] and of mRNA at some other sites, e.g., nasal mucosa [1115] and placenta [381].

9.7.14.2 Regulation

A lung-specific factor (LSF) protein has been reported to bind in the -152 to -182 5′-region of the

gene to yield the preferential expression in lung [1116]. The factors Sp1 and Sp3 have also been implicated in the expression of P450 2F1 [1117].

Metabolites of the P450 2F1 substrate 3-methylindole have been reported to induce P450 2F1 by a non-AhR mechanism [1118].

9.7.14.3 Genetic Variation

Polymorphisms have been reported in the CYP2F1 gene [1119, 1120]. The http://www.cypalleles.ki.se website currently shows eight alleles, with two frameshift variants and five coding region variants. The most frequent (*2A) is a frameshift and does not lead to a functional P450 2F1 [1119].

9.7.14.4 Substrates and Reactions

Several model fluorescent substrates have been used with P450 2F1 [1121], but most of the interest in P450 2F1 has been in regard to its ability to activate several potential toxicants and carcinogens, including 4-ipomeanol [1121], 3-methylindole [1122, 1123], styrene [1124], and naphthalene [1125].

9.7.14.5 Structure

No crystal structures have been reported. At least one homology model has been published [1126].

9.7.14.6 Inhibitors

The substrate 3-methylindole has been also reported to be a mechanism-based inactivator of P450 2F1 [1127].

9.7.14.7 Clinical Issues

Clinical issues have not been considered. Although functional polymorphisms have been reported [1120] and potential carcinogens can be activated by P450 2F1 (*vide supra*), epidemiological reports have not appeared.

9.7.15 P450 2J2

9.7.15.1 Sites of Expression

P450 2J2 is generally considered an extrahepatic P450. The highest level of expression is in the heart, but expression is also seen in skeletal muscle, placenta, small intestine, kidney, lung, pancreas, seminal vesicles, leukocytes, and brain [1128–1136]. The protein has been detected in human liver microsomes using LC–MS [635], although at a low level in one study [55]. High levels of P450 2J2 are expressed in adult human primary cardiomyocytes [1129]. Varying levels of P450 2J2 are expressed in human fetal tissues [1137]. P450 2J2 has also been reported to be expressed at higher levels in some tumors [833].

9.7.15.2 Regulation

The general consensus in the literature is that P450 2J2 is not very inducible [833, 1129]. Totah's laboratory reported a twofold induction of P450 2J2 mRNA by rosiglitazone in human primary cardiomyocytes [1129]. It has been reported that some regulation of P450 2J2 occurs through an AP-1 site and with microRNA let-7B [833, 1138–1140].

9.7.15.3 Genetic Variation

At least ten genetic variants of the *CYP2J2* gene have been reported (http://www.cypalleles.ki.se). Of the six alleles examined (other than wild type), five resulted in lower activity [1141]. Racial differences have been reported [1142].

Associations have been considered for a number of disease states, including diabetes [1143], hypertension [1144, 1145], ischemia [1146], and myocardial infarction [1147]. Other disease states have been considered with P450 2J2 in animal models.

9.7.15.4 Substrates and Reactions

The major endogenous substrate known for P450 2J2 is arachidonic acid, which is converted to all four epoxides (EETs) [1128]. These epoxides have a variety of biological activities and are a considerable source of interest (see also P450 2C9, Sect. 7.9.4).

P450 2J2 has also been found to be rather proficient in the oxidation of a number of drugs, including terfenadine [1129, 1148], ebastine [1149], astemizole [1150, 1151], hydroxyebastine and carebastine [1152], eperisone [1153], vorapaxar [1154], amiodarone [1155], albendazole and fenbendazole [833], thioridazine, me-

soridazine, danazol [1148], apixaban [1156], and some model substrates [1157]. With these drugs, it is not clear how much the generally extrahepatic metabolism of these contributes to the overall clearance, but in some cases local metabolism may be important.

9.7.15.5 Structure

No crystal structures have been reported. Some homology models have been proposed [1157, 1158].

9.7.15.6 Inhibitors

Several inhibitors of P450 2J2 have been synthesized, some with sub-μM K_I values [1157, 1159, 1160]. One of the goals is to inhibit P450 2J2 in tumors [1159]. Of the available drugs, danazol was the most selective and potent inhibitor (K_I 20 nM for inhibiting artemizole oxidation) [1155].

To date, there appear to be no reports of issues of drug–drug interactions due to inhibition.

9.7.15.7 Clinical Issues

As indicated earlier, there have not been any issues of drug–drug interaction with P450 2J2, and exactly how much this P450 contributes to overall drug clearance is unknown.

The major issue with P450 2J2 is its role in endogenous metabolism (i.e., arachidonic acid oxidation) and the etiology of several diseases, including hypoxia [1161], cardiotoxicity [1162, 1163], coronary artery disease [1164–1167], myocardial infarction [1168–1170], atherosclerosis [1171], hypertension [1172, 1173], asthma [1174], stroke [1169, 1175], hyperhomocysteinemia [1176], diabetes [1177], preeclampsia [1178], Crohn's disease [1179], and others [1130, 1180–1182].

9.7.16 P450 2R1

9.7.16.1 Sites of Expression

In the last edition of this chapter [149], nothing was known about P450 2R1. Today, this P450 is recognized as a major contributor in vitamin D metabolism and a three-dimensional structure is available [1183–1185].

Russell and his associates [1183] first cloned mouse P450 2R1 in a search for a liver microsomal vitamin D_3 25-hydroxylase. The mRNA is abundant in liver and testis of mice and was also identified (mice) in kidney, brain, epididymis, skin, heart, muscle, and spleen [1183]. In humans, a similar mRNA profile was reported [1186], with the highest levels in testis, followed by pancreas, and then the tissues reported by Cheng et al. [1183], including liver. Thus, P450 2R1 mRNA is expressed in many tissues. Protein detection has not been reported.

9.7.16.2 Regulation

Almost all of the work on regulation comes from cell culture systems. DNA methylation levels have been reported to predict variations in response to vitamin D [1187]. In a prostate cancer cell line (LNCaP cells) and skin fibroblasts, calcitriol suppressed P450 2R1 mRNA levels [1188]. The drug efavirenz suppressed P450 2R1 in fibroblasts but not LNCaP cells.

9.7.16.3 Genetic Variation

With the finding that P450 2R1 is a major vitamin D 25-hydroxylase [1183], considerable effort has been put into establishing the relationships of genetic variations. Shortly after the report that P450 2R1 is a vitamin D 25-hydroxylase [1183], Russell's group also reported that a patient with low circulating levels of 25-hydroxyvitamin D had an L99P change, which was associated with the defect [1189]. Surprisingly, no other polymorphisms have been entered in the http://www.cypalleles.ki.se site as of this writing. A GWAS of circulating vitamin D levels also identified an SNV in *CYP2R1* [1190].

However, a number of studies (not all cited here) have been done, and not all associated diseases under investigation are linked with the variation (see Clinical Implications, *vide infra*).

9.7.16.4 Substrates and Reactions

The only reaction attributed to P450 2R1 is the 25-hydroxylation of both vitamin D_2 and D_3 [1191].

A number of animal and human P450s (at least six) have been reported to catalyze vitamin D 25-hydroxylation, including P450s 2R1,

27A1, 3A4, 2J3, 2J2, 2D25, and 2C11 [1192]. In mice, a *CYP2R1* knockout lowered the level of 25-hydroxyvitamin D in serum by 50% and a *CYP27A1* deletion had no further effect [1184]. At least in mice, there may be another as yet unknown vitamin D 25-hydroxylase [1184]. Of the human P450 enzymes examined, P450 2R1 had >20-fold higher catalytic efficiency than any other P450 in vitamin D_3 25-hydroxylation [1191].

9.7.16.5 Structure

A crystal structure of P450 2R1 with bound vitamin D_3 has been reported [1185]. Cyclodextrin (used to solubilize the ligand vitamin) was present near the F–G loop. Vitamin D_3 was bound at a channel between the G- and I-helices and the B^1 helix/B–C loop, in an elongated conformation. The C-25 carbon distance to the heme iron was 6.5 Å, slightly longer than might be expected. However, this distance might change with the redox state or binding of P450 2R1 to accessory enzymes.

9.7.16.6 Inhibitors

Apparently, no inhibitors of P450 2R1 have been reported.

9.7.16.7 Clinical Issues

The major issue is vitamin D-dependent rickets, a rare autosomal recessive disease associated with low levels of activated vitamin D_3. This is the disease associated with the L99P variant by Cheng et al. [1189].

Since then a number of studies have been done to associate P450 2R1 with other diseases, including asthma [1193, 1194], diabetes [1195], multiple sclerosis [1196], and cancers [1197, 1198].

9.7.17 P450 2S1

9.7.17.1 Sites of Expression

P450 2S1 was discovered by Ingelman-Sundberg's group [1199] in searching databases. mRNA and protein blotting work indicate highest levels of expression in trachea, lung (and fetal lung), stomach, small intestine, and spleen. mRNA expression was also detected in colon, appendix, liver [1200], kidney, thymus, brain (substantia nigra), peripheral leukocytes, and placenta [1199, 1201]. Recently the protein was detected in human liver [635].

9.7.17.2 Regulation

Rivera et al. [72] reported that both mouse and human P450 2S1 mRNA transcripts are inducible by TCDD in cell culture, in a mechanism involving the AhR. Interestingly, induction is not seen in rats [1202]. Downregulation by corticosteroids in cell culture has been reported [1203].

9.7.17.3 Genetic Variation

Genetic variation appears to be extensive, with at least 13 alleles reported [1204, 1205] (http://www.cypalleles.ki.se). Most of these are outside of the coding region, and in no case have any resulting phenotypic changes been identified.

9.7.17.4 Substrates and Reactions

The identification of substrates for human P450 2S1 has been somewhat controversial. Reports of two oxidations—retinoic acid and naphthalene [1206, 1207]—have not been repeatable, at least with an *E. coli* recombinant enzyme [350, 1208]. Bui et al. [1209] reported that P450 2S1 could not be reduced by NADPH-P450 reductase, but this was disproven in a series of reduction reactions [263, 1208, 1210].

Bui et al. [1209] reported "peroxygenase"-type reaction of P450 2S1 with hydroperoxides. Such reactions have long been known in the P450 field [1211, 1212], but their physiological relevance has never been established. In these peroxygenase reactions, a number of polycyclic hydrocarbons and aflatoxin B_1 were substrates [1209]. However, one point that should not be dismissed is that these compounds were oxidized in cells in which P450 2S1 was transfected [1213], regardless of the mechanism. It is conceivable that the N-terminal modification used to express P450 2S1 might alter its catalytic selectivity, but the expressed form is definitely capable of accepting electrons from NADPH-P450 reductase [1210]. Other substrates for P450 2S1 include some aryl-

hydroxylamines, which are reduced to arylamines [263, 1214] (the corresponding arylamines were not substrates for oxidation). Some *N*-oxides are also reduced by P450 2S1 [1208, 1210].

Surprisingly, then, P450 2S1 is left without catalyzing any typical mixed-function oxidations, only reductions and peroxygenations. It seems highly unlikely it would only catalyze reductions. A metabolomic search of lungs from *CYP2S1*$^{(-/-)}$ mice revealed the accumulation of two molecules, taurocholic acid and tauro-β-muricholic acid, but only in female mice (Xiao, Y., Ding, X., and Guengerich, F.P., unpublished). Neither compound was found to be a substrate for human P450 2S1 nor were any of the precursors, so that a number of other explanations must be considered. Nevertheless, the relevance to any particular catalytic selectivity is unknown.

(Human) P450 2S1 was found not to appreciably activate any of a battery of procarcinogens tested [350].

9.7.17.5 Structure

No structure has been reported. One homology model has been published [1209].

9.7.17.6 Inhibitors

No inhibitors have been reported, in that definitive oxidations have not been identified.

9.7.17.7 Clinical Issues

The only clinical issue involves searches for association of cancer and respiratory diseases with genotype [1215, 1216].

9.7.18 P450 2U1

9.7.18.1 Sites of Expression

Essentially all of the expression reports have been at the mRNA level. P450 2U1 mRNA expression has been reported in brain and thymus [1217, 1218]. Some expression was also detected in other tissues, including heart, kidney, liver, lung, testes, and leukocytes [1217]. In the brain, the highest level of mRNA was in the cerebellum, as well as limbic structures and cortex, plus cerebellum, olfactory bulbs, and pons and medulla [1217]; see also [1219]. Another site of expression is white adipose tissue [1220]. P450 2U1 is also expressed in skin [1221].

9.7.18.2 Regulation

Relatively little is known about regulation of P450 2U1, other than what might be inferred from aspects of tissue localization (*vide supra*). P450 2U1 mRNA was upregulated in leukocytes following trauma, for unknown reasons [1222].

9.7.18.3 Genetic Variation

Genetic variation of P450 2U1 has been reported in a French population, with four variants reported [1223]. All of these four variations are outside of the protein coding region.

9.7.18.4 Substrates and Reactions

Chuang et al. expressed P450 2U1 in a baculovirus-based system and reported the ω- and ω-1 hydroxylation of arachidonic acid [1217]. Other long-chain fatty acids were oxidized (sites not identified) but short-chain fatty acids were not. Substrates included arachidonic, palmitic, palmitoleic, stearic, and vaccenic acids, plus eicosapentaenoic and docosahexaenoic acids. No kinetic parameters were reported [1217].

A metabolomics-based search for P450 2U1 substrates revealed arachidonic acid and also *N*-arachidonoylserotonin as substrates [1224]. The site of oxidation of *N*-arachidonoylserotonin was identified as the C-2 of the indole ring [1224]. *N*-Arachidonoylserotonin, an inhibitor of fatty acid amide hydrolase [1225], was shown to be present in human brain, and the oxidation at the C-2 site attenuated its ability to inhibit the hydrolase [1224].

9.7.18.5 Structure

No information is presently available.

9.7.18.6 Inhibitors

No information about inhibitors is presently available.

9.7.18.7 Clinical Issues

At the present time, there are no clinical issues regarding P450 2U1. The only clinical issues in-

volve the potential of P450 2U1 as a tumor marker [1226]. A variant has been associated with complicated forms of hereditary spastic paraparesis [1227].

9.7.19 P450 2W1

9.7.19.1 Sites of Expression

mRNA searches showed little expression in most tissues [350, 1228] but expression in colorectal tumors [1228]. However, the protein has also been detected in human liver [635].

9.7.19.2 Regulation

P450 2W1 has been shown to be regulated by gene methylation [185]. The protein has also been reported to be glycosylated in human embryonic kidney (HEK)-293 cells and to have inverted endoplasmic reticulum topology [185].

9.7.19.3 Genetic Variation

Several reports have appeared on the genetic variation of P450 2W1 [1229–1232]. The http://www.cypalleles.ke.se website lists seven known alleles, five of which lead to coding changes (effects are unknown). One of the issues is potential relationship to colon cancer prognosis.

9.7.19.4 Substrates and Reactions

Although P450 2W1 could probably still be considered an "orphan" P450 (Table 9.1), a number of catalytic activities have now been ascribed to it. P450 2W1 activates a number of procarcinogens, including PAHs, aflatoxins, and aryl- and heterocyclic amines [350, 1233]. A cancer chemotherapeutic agent, AQ4N, is reduced by P450 2W1 [1208]. P450 2W1 also activates several cancer chemotherapeutic agents by oxidation, including aryl benzothiazoles [1214, 1234] and duocarmycin analogs [1235].

A metabolomic search for endogenous substrates for P450 2W1 revealed lysolecithins [1236]. Hydroxylation and epoxidation at the internal carbons of the fatty acids were observed, and the reaction occurred with other monoacyl (but not diacyl) glycerophospholipids [1236]. Other reported substrates are indole, 3-methylindole, and chlorzoxazone [1237]. Only very low catalytic activity towards arachidonic acid is observed [350, 1228, 1237].

9.7.19.5 Structure

A homology model of P450 2W1 has been published [1238].

9.7.19.6 Inhibitors

No inhibitors of P450 2SW1 have been reported.

9.7.19.7 Clinical Issues

The only clinical issues reported relevant to P450 2W1 relate to the possibility of P450 2W1 expression as a cancer marker [1230, 1239, 1240].

9.7.20 P450 3A4

P450 3A4 is the most abundant P450 in the human body (e.g., Figs. 9.2 and 9.3) and has a dominant role in drug metabolism (Fig. 9.1b). Some of the earliest preparations of human P450 [9, 10] were retrospectively found to be P450 3A4. Two approaches led to an extensive characterization. Watkins et al. [1241] isolated a P450 from human liver using immunochemical cross-reactivity with what is now recognized as a rat subfamily 3A P450. This laboratory isolated an enzyme from human livers that catalyzed the oxidation of the hypotensive dihydropyridine drug nifedipine [16]. cDNA cloning yielded sequences corresponding to *CYP3A3* [1242] and *CYP3A4* [1243]. (The former differed from *CYP3A4 at* 14 sites and could be considered a rare allele, although it has not been reported again [1244–1246] and originally came from the same single-liver cDNA library as the *CYP3A4* clone; *CYP3A3* has accordingly been dropped from the nomenclature and earlier references to this should probably be considered to indicate P450 3A4.)

Subsequently studies with microsomes, antibodies, and purified P450 3A4 quickly indicated that nifedipine was not the only substrate; other substrates included other dihydropyridines [1247], steroids [16, 1248], quinidine [97], the oral contraceptive 17α-ethinylestradiol [26], and the carcinogen aflatoxin B_1 [29]. With more stud-

ies and the application of recombinant systems, the repertoire of substrates expanded rapidly [1249].

9.7.20.1 Sites of Expression

P450 3A4 is the most abundant P450 in human liver and in the small intestine. The average fraction of the total P450 in liver accounted for by P450 3A4 has been estimated to be 25–30% [52] (Figs. 9.2 and 9.6); in small intestine, the fraction attributed to P450 3A4 is even higher (Fig. 9.3). A study with the selective inhibitor gestodene, which destroys P450 3A4, indicated that P450 3A4 can constitute 60% of the total hepatic P450 [1250]. Several estimates have been made of the absolute amount of P450 3A4 (Fig. 9.2b, c, and d). One estimate with a pool of Japanese samples was 64 pmol P450 3A4/mg protein, but analysis of nine individual samples in the same laboratory yielded a mean of 9 (pmol P450 3A4/mg microsomal protein, range 1–28) [54]. Another laboratory reported a mean of 68 (pmol P450 3A4/mg microsomal protein, range 10–262) [55].

P450 3A4 is also expressed in some extrahepatic tissues, including lung [382, 1251], stomach, colon [382], brain [1252], and adrenal (weak) [1253]. P450 3A4 has not been reported to be expressed in kidney, prostate, testis, or thymus but other subfamily 3A P450s are [1253]. P450 3A4 expression has been reported in brain at both the mRNA and protein levels, particularly in the cortex, neurons, and blood–brain barrier endothelial cells [1252, 1254, 1255]. This location is of relevance regarding not only drug metabolism of neurochemical drugs but also metabolism of endogenous chemicals there, e.g., morphine (Sect. 7.20.4, *vide infra*; Fig. 9.17). The literature is mixed on whether expression occurs in peripheral blood lymphocytes or not [1253, 1256].

P450 3A4 is expressed in some tumors, although the literature is very mixed as to reports of levels being lower or higher than the surrounding tissue [1257–1259].

A significant gender difference in P450 3A4 expression does not appear to occur [52, 64] (although one report indicated a difference [65]), and some apparent pharmacokinetic gender differences may be attributable to P-glycoprotein not P450 3A4 [66]. In fetal liver, P450 3A7 is the most abundant form and P450 3A4 expression is very low [174, 1260]. P450 3A4 expression increases rapidly after birth and reaches 50% of adult levels between 6 and 12 months of age [1260]. Although many general regulatory concerns have been expressed about additional safety margins for children with drugs and other chemicals, the evidence in this case indicates that P450 3A4 activity levels in infants are slightly higher than in adults [1260]. Other studies concur that there is a marked development of P450 3A4 (switch from P450 3A7 expression fetal period) following birth and increase during the first year of life [1261], with relatively little change after childhood [64].

9.7.20.2 Regulation

The *CYP3A4* gene is at chromosome 7q22.1 [1262]. Although 3A subfamily enzymes were long known to be inducible in animals [1263] and considerable literature existed on the in vivo induction of many activities by barbiturates and macrolide antibiotics (e.g., rifampicin) [2], early demonstrations of inducibility were indirect but some progress was made [1241]. A general correlation between enzymes and mRNA levels could be shown in human liver samples [1242, 1244]. Defining the mechanism of regulation was difficult [1264], to some extent because of the difficulty in finding appropriately responsive cells to utilize the *CYP3A4* gene and vector constructs derived from it. Guzelian's laboratory reported that the source of liver cells was a greater issue than the *CYP3A* regulatory region in comparing interspecies differences in *CYP3A* gene regulation [1265], and this result can now be rationalized in the context of new knowledge about receptors (*vide infra*).

Although most *CYP3A* subfamily genes are inducible by dexamethasone, the classic glucocorticoid receptor was shown not to be involved in rat liver [1266]. In early 1998, Maurel and his associates reported that the macrolide antibiotic rifampicin acted as a nonsteroidal ligand and agonist of the human glucocorticoid receptor, providing a possible mechanism for regulation and a difference with the rodent systems [1267].

The interpretation of these conclusions was questioned by Ray et al. [1268].

Shortly thereafter, Kliewer's group characterized the human homologue of mouse PXR, which bound steroids and interacted with *CYP3A* subfamily genes in the manner expected for a major regulatory influence [1269, 1270] (some literature also refers to the human PXR as "SXR"). This member of the steroid receptor family "orphan" group (PXR) interacted with barbiturates, steroids (including dexamethasone), statin drugs, macrolide antibiotics, and some organochlorine pesticides [1270, 1271].

Knowledge of PXR and its cognate binding site has led to the development of PXR receptor and reporter assays to screen for P450 3A4 induction with new drug candidates [1272–1274]. The discovery of PXR suggested that alleles of this receptor might be responsible for the variable inducibility in different individuals. However, the PXR SNVs found to date have not been found to control P450 3A4 induction [1275]. The regulation of *CYP3A4* expression is more complicated than simple loading of activated PXR (e.g., Fig. 9.13), as suggested by Kliewer's early work showing the roles of coactivators [1269, 1270]. However, the glucocorticoid-mediated induction of P450 3A4 is mediated by elements in addition to the canonical PXR site [1276, 1277]. Some compounds (e.g., ketoconazole) suppress *CYP3A4* gene expression, apparently via binding to the PXR and interaction with "corepressors" (NCoR, SMRT) [1278]. CAR (see Sect. 6.7.2) appears to interact with the *CYP3A4* gene at the PXR site to cause induction [1279]. Further, there is evidence that 1*α*,25-dihydroxyvitamin D_3 (see Sect. 6.53) also controls the transcription of P450 3A4 [1280]. This effect is mediated through the VDR [551], which has similarity to PXR and CAR in the steroid receptor superfamily. Further, kinases have been shown to modulate the induction of P450 3A4 via VDR in Caco-2 cells [1281].

Other factors also contribute to P450 3A4 regulation. Among these are C/EPP*α*, DBP [1282], and HNF-4*α* [1283]. Interleukin-6 has been reported to downregulate P450 3A4 through translational induction of the repressive C/EBP*β*-LIP protein [1284]. Thus, the transcriptional regulation of P450 3A4 expression centers on PXR but involves many other aspects. A systematic transcriptomic analysis of the regulation of human P450s, including P450 3A4, has been published, based on pathway analysis in human liver samples [64, 1285].

Regulation of P450 3A4 expression has been reviewed by Schuetz [1286]. The P450 3A4 gene is somewhat unique in having an upstream proximal ER6 element, with xenobiotic response enhancer module (XREM) and constitutive liver enhancer module (CLEM) [1287]. The novel enhancer CLEM4 is important, and HNF-1*α*, HNF-4*α*, upstream stimulatory factor (USF) 1, and Ap-1 all interact with CLEM-4 [1288]. It is also polymorphic. HNF-4*α* determines PXR- and CAR-mediated induction of P450 3A4 [1289]. Nuclear factors (e.g., VDR) can compete with PXR for binding to its cognate site [1290]. PPAR*α* has also been reported to regulate P450 3A4 [1291].

Based on results obtained with endometrial samples, it has been postulated that estrogens upregulate P450 3A4 [1292]. In addition, Wolbold et al. [1293] reported twofold higher levels of P450 3A4 in livers from women than men in a collection of 94 samples. However, this gender dimorphism has not been observed in other studies except for Schirmer et al. [65], which was only seen when testosterone 6*b*-hydroxylation activity was considered (but not when midazolam was the test substrate) and was not statistically significant [64].

Another aspect of P450 3A4 regulation involves degradation. TAO, erythromycin, and some related amine macrolide antibiotics form "metabolite complexes" (C-nitroso:iron (R–N=O:Fe)) and inactive protein accumulates [1294, 1295]. These studies have relevance to in vivo P450 3A4 inhibition by these drugs.

Degradation of P450 3A4 appears to be degraded by a ubiquitin-linked pathway [336]. Correia and her associates also reported that protein kinase C-modified P450 3A4 at Thr-264 and Ser-420; the relevance of these phosphorylations to ubiquitin-linked degradation is yet unknown [1296].

Phosphorylation of P450 3A4 has been reported in liver samples [297]. The effect on catalytic activity is not known. Phosphorylation (Thr-264, Ser-420, Ser-478) is also important in ubiquitin-dependent proteasomal degradation [1297], which involves gp78 and CHIP E3 ligases [1298]. Conformational phosphodegrous (negatively charged patches) have been considered for (ubiquitin) E2/E3 recognition [1299]. The NFκB pathway has also been considered to interact with proteasomal degradation in regulating the stability of the P450 3A4 protein [1300].

Hughes et al. [1301] reported that progesterone receptor membrane component 1 (PGTMC1, or Dop1) binds and regulates (human) P450 3A4, based on work with a yeast model. However, in studies in mammalian cell culture, downregulation of PGTMC1 did not affect expression or localization of P450 3A4 [1302]. Transfection of PGRMC1 along with P450 3A4 resulted in the inhibition of P450 3A4, and this inhibition was relieved by increased expression of NADPH-P450 reductase.

9.7.20.3 Genetic Variation

The issue of genetic variation is considered in the context of attempts to explain the population variability in P450 3A4 activity, which does not show truly modal distribution [1303].

At least 43 alleles of P450 3A4 are known, and an additional four SNVs have yet to be characterized with regard to haplotype (http://www.cypalleles.ki.se). The SNVs and other variants identified have not yet shown much relationship to catalytic activities [1304–1310].

Some of the variants have impaired function [1311, 1312]. The *17 allele (coding for an F189S change) had < 1 % of the normal catalytic activity [1313]. Polymorphisms in transcription factors and other regulatory proteins can influence P450 3A4 expression [1314].

Klein and Zangar [1315] have reviewed the contributions of various genetic components in the context of the overall variation in P450 3A4.

9.7.20.4 Substrates and Reactions

Analysis of the catalytic activity of P450 3A4 and other subfamily 3A P450 enzymes is not always easy to assess because of nuances about the effects of the membranes and other proteins. Wrighton examined P450s 3A4, 3A5, and 3A7 under identical conditions and concluded that P450 3A4 is generally more catalytically active than 3A4 or 3A7 towards all substrates examined [1316].

9.7.20.4.1 Substrates

P450 3A4 contributes to the metabolism of ~50 % of the drugs on the market or under development (Fig. 9.1b). For lists, see Table 9.5 and Rendic [51]. Many of these are important drugs such as simvastatin (Zocor®) and some other statins [1317], the prostate hypertrophy inhibitor finasteride (Proscar®/Propecia®) [1318], the immune suppressant cyclosporin [20, 1319], protease inhibitors such as indinavir [1320], and sildenafil (Viagra®) [1321].

In the course of these reactions, P450 3A4 catalyzes some atypical reactions [887], including desaturation [1317], oxidative carboxylic acid ester cleavage [1322], and oxidation of a nitrile to an amide [1323]. An unexpected reaction encountered in this laboratory was the oxidation of alkylphenyl ether nonionic detergents, which have been commonly used in enzyme purification [537] and also have some medical and industrial applications [1324]. Methylene hydroxylations yield hemiacetals, which break down to shorten the chains [1324].

One of the classic (and fastest) reactions catalyzed by P450 3A4 is testosterone 6β-hydroxylation [16]. However, the physiological significance of this and several other steroid hydroxylations [1248] is unclear. The significance of P450 3A4 in physiology may be questioned, given its variability (Fig. 9.5). However, some contributions are possible and may be suggested from recent work. Cholesterol is oxidized by P450 3A4 to 4β-hydroxycholesterol, a major circulating oxysterol [1325, 1326]. P450 3A4 also catalyzes the 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol [1327, 1328]. The product is a potent PXR agonist, and this system might function as an autoregulatory pathway (i.e., excess triol activates PXR and P450 3A4, which reduces the level of triol [1329]).

P450 3A4 also functions in the metabolism of cancer chemotherapeutic drugs. In addition, attention has been given to activations of drugs and chemical carcinogens. P450 3A4 activates the ER antagonist tamoxifen to produce DNA adducts (Fig. 9.10) [1330]. Another example of carcinogen activation involves aflatoxin B_1, which undergoes both a detoxicating 3α-hydroxylation and formation of the highly mutagenic 8,9-*exo*-epoxide [29, 1331, 1332]. Some other carcinogen substrates of P450 3A4 are listed in Table 9.8.

One of the issues with P450 3A4 is which reaction provides the most appropriate index of activity, both in vitro and in vivo. Historically nifedipine oxidation and testosterone 6β-hydroxylation were among the first activities identified [16] and are still used in vitro [158]. Midazolam 1′-hydroxylation has also been used extensively [158], in part because of its acceptance for in vivo assays.

Some higher-throughput fluorescence assays were also developed and gained commercial appeal [1333, 1334]. One issue regarding these and also several other P450 3A4 reactions is that they show variable effects of added chemicals, i.e., one compound may inhibit a certain P450 3A4 reaction but stimulate another. Chauret et al. [1335] reported a fluorescence reaction that behaves in a very similar way to testosterone 6β-hydroxylation. Houston has examined the behavior of P450 3A4 probe substrates in vitro and grouped them into two categories. Although all of these reactions are catalyzed by P450 3A4, they have been categorized into two groups by their behavior in the presence of other compounds, as mentioned above [1336]. One group includes testosterone, cyclosporin, and erythromycin. The second includes midazolam, triazolam, dextromethorphan, and diazepam. Terfenadine fits in either group, and nifedipine seemed to have properties unique from both groups [1336].

The ambivalence about the variability of probe drugs is even greater for in vivo human experiments than in vitro, as one might expect. A number of reactions have been used, including nifedipine oxidation [1337], erythromycin *N*-demethylation [1338], lidocaine oxidation [1339], dapsone *N*-hydroxylation [1340], midazolam 1′-hydroxylation [1341], and quinine 3′-hydroxylation [1342]. In most cases, the test drug is administered orally for convenience, except for some uses of erythromycin and midazolam (i.v.). The ratio of (endogenous) urinary 6β-hydroxycortisol to cortisol has also been used to assess P450 3A4 function [1343]. Many of the assays reflect the activity of P450 3A4 in the small intestine, particularly with the drugs administered orally. The erythromycin breath test (exhaled CO_2 produced from the HCHO released in the reaction) is generally used to estimate hepatic P450 3A4 and has been used as an aid in selecting cyclosporin doses for liver transplant patients [1344]. The lack of correlation of these indicators is a problem in the practical analysis of drug interactions [1345–1347]. Some of the discrepancies are probably inherent in the nature of P450 3A4 itself (i.e., see in vitro assays, *vide supra*). Other issues involve the lack of coordinate regulation of hepatic and intestinal P450 3A4 [1348] and the activity of P-glycoprotein [1349] which shows some overlap in regulation patterns with P450 3A4 [1350] and influences the availability of substrates to P450 3A4 in both small intestine and liver.

The substrates of most interest with P450 3A4 are drugs, steroids, and carcinogens. It is very clear that P450 3A4 is a major factor in drug metabolism (Figs. 9.1b, 9.2, and 9.3). P450 3A4 catalyzes many steroid reactions, although the physiological significance of these remains to be established. P450 3A4 is also able to activate many procarcinogens (Fig. 9.10) [99], although the impact on human cancer remains to be established.

P450 3A4 is involved in the oxidation of cholic acid to 3-dehydrocholic acid and of chenodeoxycholic acid (CDCA; 6α-hydroxylation) [1351]. P450 3A4 hydroxylates cholesterol at the 4β-position, and this product accumulates and can be of use as a noninvasive marker of P450 3A4 function [1352–1354]. Cholesterol is also hydroxylated at the 25 position by P450 3A4 [1355, 1356].

P450 3A4 has also been demonstrated to catalyze testosterone 1β-hydroxylation [1357] and progesterone 21-hydroxylation [1358]. P450

3A4 has long been known to catalyze estradiol 2- (and some 4-) hydroxylation [16]; more recent studies with transgenic mice suggest that P450 3A4 may have an important physiological role in catalyzing this reaction in vivo [1359].

In addition to the list of major drugs for which P450 3A4 has a major role (Tables 9.5, 9.6, 9.7, and Fig. 9.1b), the enzyme has more recently been shown to have roles in the oxidation of thalidomide [1360, 1361], and tamoxifen (α-hydroxylation) [1362].

P450 has also been demonstrated to play important roles in the biosynthesis of endogenous morphine in mammals, catalyzing both (1) the cyclizations of (*R*)-reticuline to salutaridine [905] and (2) the elusive O^6-demethylation of thebaine involved in the latter stages of morphine synthesis [906] (Fig. 9.17). With this, a minimal scheme can be proposed with P450 enzymes capable of all oxidative steps in the pathway.

9.7.20.4.2 Catalytic Mechanism

The mechanism of P450 3A4 has been studied extensively, and several aspects of it bear discussion (along with structure considerations, Sect. 7.20.5) before considering the issue of cooperativity. The basic P450 catalytic scheme is actually a rather minimal scheme. Studies with substrates and inhibitors provided evidence that substrate binding is a multistep kinetic process [217, 218], as corroborated by others [219]. The evidence for multiple occupancy of P450 3A4 (Sect. 7.20.5, *vide infra*), coupled with the multistep binding, makes the process difficult. Sligar and his associates have shown that the oxidation–reduction potential of P450 3A4 is lowered by at least some substrates [1363], and the presence of substrate is known to facilitate rates of reduction of ferric P450 3A4 [1364, 1365]. The FeO_2^{2+} complex has been observed (stabilized in the presence of substrate) but is less stable than in several other P450s and degrades rapidly [1366, 1367]. Some, but not all, P450 3A4 reactions are stimulated by the presence of cytochrome b_5 [1365]. Two surfaces of cytochrome b_5 have major and minor roles in interactions with P450 3A4 [1368]. Electron transfer is not required for the stimulatory role of cytochrome b_5 (with P450 3A4), in that apo-cytochrome b_5 (without heme, devoid of electron transfer capability) is also effective [1369, 1370].

One point that can be made here (but that applies to many P450s) is that they exist, in part, in the ferrous state in the cell [1371]. Thus, the ferric state is not necessarily the starting point in the catalytic cycle.

Deuterated testosterone has been used to probe the catalytic mechanism of P450 3A4 [1372] (Fig. 9.19). Abstraction of the 6β hydrogen of testosterone is highly stereoselective, with the oxygen rebound also going only to the β position. The use of both 6-deuterated and 6-tritiated testosterone led to the conclusion that the 6β-hydroxylation step has a high intrinsic kinetic deuterium isotope effect, which is considerably attenuated in the steady state [1372]. The conclusion is that steps other than C–H bond breaking limit rates of the steady-state reaction.

More recently, P450 3A4 was also shown to oxidize 4,5-dihydrotestosterone, a more potent androgen that differs only from testosterone in the pucker of the A-ring (Fig. 9.20). The sites of hydroxylation were the two axial methyls (C-18, C-19; Fig. 9.20), which is surprising on the basis

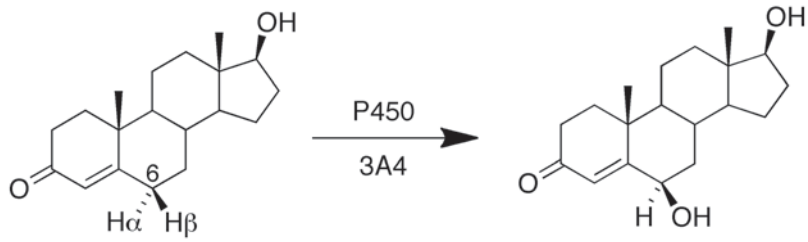

Fig. 9.19 Stereoselective removal of 6β-hydrogen from testosterone by P450 3A4 [1372]

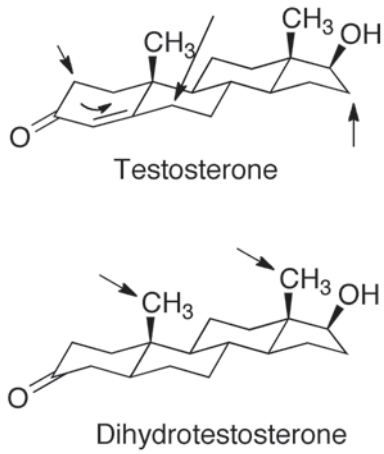

Fig. 9.20 Regioselectivity of oxidation of testosterone and dihydrotestosterone by P450 3A4 [1357, 1373]

of both chemical reactivity and modeling predictions [1373].

9. 7.20.4.3 Cooperativity

At the outset, cooperativity of P450s was regarded as a curiosity, but today there is interest in practical settings, as reviewed by Obach [1374]. Both heterotropic and homotropic cooperativity have been observed with several human (and other P450s), although it has been most reported with P450 3A4 over the past 20 years [1375]. For a review of the mechanistic issues with P450 3A4, see Sevrioukova and Poulos [1376].

Although there are older examples of in vivo heterotropic cooperativity [202], it is difficult to assign these to particular P450s. The results of Tang and Stearns with quinidine and warfarin in animals are probably attributable to subfamily 3A P450s [207]. Evidence for heterotropic oxidation of thalidomide in a transgenic P450 3A4 mouse has been presented [1377]. Also, midazolam oxidation could be stimulated by a drug candidate—5-(4-fluorobenzyl)-2-((3-fluorophenoxy)methyl)-4,5,6,7-tetrahydropyrazolo[1,5-*a*] pyrazine—that also had (in vivo) enhancing effects in rats [1378].

Suggestions of multiple occupancy of the active site of P450 3A4 were made in the 1990s [205, 211, 212]. However, dual occupancy was not definitively demonstrated until the X-ray crystallography work of Ekroos and Sjogren [213] appeared. A number of kinetic and spectroscopic measurements were analyzed and estimates of the number of ligands included in the active site of 2–4 were made using various models [1379–1384]. Cytochrome b_5 has been reported to induce P450 3A4 substrate cooperativity [1385].

To summarize the cited literature (and much more for which space was not available), the evidence is in favor of cooperativity involving multiple occupancy within the active site (of P450 3A4 in this case), and there is little if any evidence for a completely distinct allosteric site on the protein. Direct evidence (X-ray crystallography) exists for multiple occupancy [213], and Auclair and her associates have shown that attaching a large molecule (theobromine) to substrates not only allows catalysis but also changes the regioselectivity of oxidation [1386]. Models based on kinetic systems are very complex, particularly in light of limited information about what step(s) is rate limiting in most cases [1372] and the demonstrated complexity of ligand binding [217, 218, 1387]. Hill plot n values for cooperativity are low (<1.5) in most cases (subject to error), and artifactual sigmoidicity can be introduced simply by running low substrate concentration reactions beyond linearity. The number of variables often greatly exceeds the experimental parameters used. Another obstacle is finding a satisfactory substrate and effector, in that the patterns with different P450 3A4 ligands are rather unpredictable.

In many respects, equilibrium physical measurements could be considered most valuable. Nuclear magnetic resonance (NMR) spectra (T_1 paramagnetic relaxation) were used to probe cooperativity of midazolam with testosterone and α-NF [1388]. Atkins and his associates [1389] used a single-molecule fluorescent approach to show "allosteric" effects of one ligand on the dissociation rate of another substrate, Nile red. Nile red is an allosteric fluorescent substrate and has utility for such studies [1390]; evidence could also be obtained for a second binding site [1391].

Another aspect and possibly another solution to the issue comes from work by Friedman using flash photolysis kinetics (of CO rebinding after

photodissociation from ferrous P450 3A4). The kinetics were multiphasic and were selectively altered by the presence of different substrates [1392]. Heterotropic effects were observed with benzo[*a*]pyrene and α-NF [1393]. The interpretation of the results is that different substrates differentially modulate these kinetics by (1) changing the P450 conformation to alter the rate, and/or (2) steric effects (of ligands) that reduce rates [1394]. Both effects are possible, although the enhancement of rates in some cases [1392] argue against the generality of the latter explanation and in favor of multiple conformations for P450 3A4 bound to various ligands. The concept advanced is that some ligands act as allosteric factors to "switch" P450 3A4 conformations [1395]. Some possibly relevant work has been done by Anzenbacherová [1396], who did pressure studies on P450 3A4 and found that the compressibility of P450 3A4 was less than that of bacterial P450 102A1; the compressibility was modified by the ligand TAO. The concept of preexisting multiple conformers of P450 3A4 is an explanation for the flash photolysis work [1392–1395] and has support in newer nonclassical approaches to general protein chemistry [1397–1399]. This view differs from the more general static "lock-and-key" view of enzyme/substrate complexes and the induced-fit theory in which enzymes are shaped by their substrates. The basic concept is that protein dynamics present an ensemble of structures of an enzyme in solution and different ligands bind to individual states depending upon their complementarity [1397–1399]. Another consideration in this discussion, somewhat related, is that there is good evidence that P450 conformations change during the course of the catalytic cycle [1400], and evidence has already been presented that different forms of P450 3A4 can differ in their binding of a ligand (e.g., ferric and ferrous) [211].

Where does all of the work to date leave us in this area? A recent review by Atkins et al. [1401] summarizes much of the work in more detail and presents a cogent analysis. Summarizing and expanding on this, there are several major possibilities to explain the observed cooperativity of P450 (and the other P450s showing this behavior), which are not necessarily exclusive: (1) a "classic" allosteric model with binding of effectors at a site that then regulates the conformation for substrate binding; (2) a relatively rigid P450 with a large active site that can accommodate two to three ligands, with the results depending on the chemical interactions of the two ligands with each other and with P450 residues; and (3) a series of preexisting conformations of P450 3A4 that selectively interact with individual ligands [1397–1399]. A general concept of induced fit is related to the third possibility, as in the phenomena already mentioned that different protein conformations exist throughout the catalytic cycle, can differ in affinities and substrate orientation, and may not be in rapid equilibria. Many steady-state kinetic schemes have been proposed but, in considering the possible origins [200, 1402], cannot be considered unique and do not provide mechanistic answers.

To return to the questions raised by Sevrioukova and Poulos [1376], there are still many unanswered questions about cooperativity, even 20 years after the first reports with P450 3A4 [204] and 45 years after the first general reports of the phenomenon with P450s [201, 203, 1403], explaining the mechanisms at a molecular level is not yet within our grasp. However, the battery of structural, spectroscopic, and other tools available is promising. There is evidence that the phenomenon may contribute to drug–drug interactions and human variability in response to molecules.

9.7.20.5 Structure

A number of site-directed mutagenesis studies on the possible roles of individual residues have been published. Phe-304 [1404] and Ala-305 [1405], in the putative I-helix, are proposed to control access to the catalytic center. Phe-304 was also implicated in the partitioning of aflatoxin B_1 oxidation (between 3α-hydroxylation and 8,9-*exo*-epoxidation) [1406]. A role for Asn-206 was also proposed in the work with aflatoxin B_1 [1406]. Leu-211 is also postulated to control the size of the active site [1407].

A number of X-ray crystal structures of P450 3A4 are now available, including the protein without a ligand [1408] and with bound metyra-

pone, progesterone [215], erythromycin, ketoconazole [213], and ritonavir and several ritonavir analogs [1387, 1409, 1410]. The active site is large (~1285 Å^3), and in the case of ketoconazole two molecules of the ligand are present, only one of which is in a position to be oxidized [213]. Another interesting aspect is the binding of progesterone at a peripheral site, 17 Å from the iron, in a position incompatible with catalysis [215]. Collectively these structures are very valuable in understanding how this enzyme handles so many reactions (*vide infra*, Fig. 9.1b). One general conclusion from all of the structural work is that P450 3A4 can use multiple conformations to accommodate different ligands, i.e., has "malleability" [1410, 1411]. Support for this malleability of P450 3A4 comes from molecular dynamics simulations, which show much more flexibility for P450 3A4 than for P450 2A6 or 2C9 [1412].

Whether the site of progesterone binding in the structure of Williams et al. [215] is relevant is an interesting question. Subsequently evidence has been presented that P450 3A4 can have initial binding to P450 3A4 prior to moving near the heme iron [217], and the peripheral site might represent this. Davydov et al. [1413] also reported a peripheral binding site for a dye (fluorol-7GA) using fluorescence energy transfer.

Cross-linking studies and mass spectrometry have been used to characterize a site of interaction of P450 3A4 with cytochrome b_5/apo-cytochrome b_5 [1414].

Numerous systems have been developed to predict sites of oxidation by P450 3A4, e.g., [926].

9.7.20.6 Inhibitors

Inhibition of P450 3A4 is a major issue in the pharmaceutical industry and the cause of a number of important drug–drug interactions (Table 9.6). A compendium of P450 3A4 inhibitors has also been compiled by Rendic [51]. Only a few other specific examples of P450 inhibitors will be mentioned here. One example of a problem leading to recall of a drug is that of terfenadine [92–95]. Inhibition of P450 3A4 is a frequent problem with drug candidates, particularly unsuspected mechanism-based inactivation, and strategies have been developed for minimizing it [1415] or making in vitro assessment as to the extent it may be an issue in vivo [1416].

The inhibition of P450 3A4 has been shown to be altered by the presence of (coding region) variations [1417].

Erythromycin and ketoconazole are two of the most established inhibitors of P450 3A4, based on clinical experience. Ketoconazole, used at ~1 μM, is probably the best established P450 3A4 inhibitor for in vitro use [85]. Another P450 inhibitor is TAO [1418], which also has clinical implications. TAO has been used as a diagnostic in vitro inhibitor of P450 3A4, although its mode of action (activation to a nitroso derivative that complexes P450 iron) requires time for the inhibition to occur.

One issue is the inhibition of P450 3A4 by grapefruit juice, first reported by Bailey [1419]. The effect was rather specific for grapefruit and a few other citrus fruits (not orange), and warning labels now include this contraindication for many drugs [1420]. Naringenin has some effect [1421], but the most active compounds appear to be the furanocoumarins bergamottin and 6′,7′-dihydroxybergamottin, which behave as mechanism-based inactivators to destroy intestinal P450 3A4 [88, 89]. The magnitude of the effect of the interaction varies with drugs, with some of the statins, buspirone, terfenadine, astemizole, and amiodaraone reported to show the greatest interactions [1420].

Many of the HIV protease inhibitors are also potent inhibitors of P450 3A4 as well as substrates in some cases [1422]. Because of the variety of drugs that AIDS patients use, the potential for interactions is considerable.

The effects of some herbal medicines on P450 3A4 have already been mentioned. In addition to P450 3A4 induction (e.g., St. John's wort), some of these materials also contain inhibitors. For instance, kava kava extracts produce kavapyrones that inhibit P450 3A4 [1423].

Oral contraceptives contain acetylenes and can be mechanistic inactivators of P450 3A4. Inactivation has been demonstrated for 17α-ethinylestradiol, the major estrogenic component of oral contraceptives [26, 1424], and several of

the progestogenic components, particularly gestodene [1250]. Because of the very low doses of these contraceptives that are used today, the effects might be expected to be small [1425], although some in vivo effects have been reported [1426, 1427].

Some chemicals and also oxidants have been shown to cause the covalent cross-linking of heme to apo-P450 [197]. Correia's group has characterized the products of the destruction of P450 3A4 with cumene hydroperoxide; the information is consistent with a dipyrrolic fragment of heme bound to fragment of the protein [1428].

Among diagnostic inhibitors used for reaction phenotyping, ketoconazole (at 1–2 μM) remains a popular choice, although it will not distinguish among individual subfamily 3A P450s [473]. Azamulin has some advantages [1429], and "CYP3cide" (PF-04981517; 1-methyl-3-[1-methyl-5-(4-methylphenyl)-1*H*-pyrazol-4-yl]-1*H*-pyrolo[3,4-*d*]pyrimidine) [1430] and ML-368 [1431] are P450 3A4-specific. Li et al. [1432] have described a P450 3A4-selective inhibitor (1-(4-imidazopyridinyl-7-phenyl)-3-(4′-cyanobiphenylurea (SR9186)) that can be utilized for inhibiting only P450 3A4 and not 3A5.

Ritonavir is one of the most potent inhibitors of P450 3A4 [1416]. A number of analogs have been compared using spectral, kinetic, and structural (crystallography) approaches [1409, 1410, 1433].

P450 3A4 is involved in the bioactivation of a number of chemical carcinogens (Fig. 9.10) [99], and one strategy for chemoprevention is to inhibit P450 3A4. A number of flavonoid inhibitors have been characterized [365]. *cis*-Terpenones have been shown to block aflatoxin B_1 cytotoxicity in vitro [1434].

Other inhibitors reported for P450 3A4 are 4-ipomeanol [1435], raloxifene [1436], and bergamottin, the active principle of grapefruit juice (*vide supra*) [1437]. In the latter two cases, the site of attachment (in the P450) has been identified.

9.7.20.7 Clinical Issues

The major clinical issues with P450 3A4 are rapid clearance (of drugs), variable bioavailability, and enzyme induction and inhibition [1438, 1439]. One of the concerns is intestinal first-pass metabolism of drugs, which usually inactivates them [1440]. One strategy to improve predictability in drug development is the use of transgenic "humanized" mice expressing P450 3A4, which have been developed using different approaches [1441, 1442]. High enzyme activity towards a drug will reduce bioavailability, and variations in levels of P450 3A4 can cause clinical problems when the therapeutic window is narrow. For instance, low cyclosporin levels will not prevent organ rejection during transplant, but high levels cause renal toxicity, so adjustment of the dose is critical [1443]. Terfenadine has a relatively wide window for use, but a few serious problems were encountered [95, 1444]. Renwick has considered population models of P450 3A4 variability and concluded that there is more interindividual variability from the oral route than i.v., which is not surprising in light of the previous discussion of the intestinal contribution to drug metabolism. A "default factor" for adults of 3.2-fold is presented, but a factor of 12(-fold) was calculated to be needed to cover 99% of the neonates as well [1445].

The effect of disease on P450 3A4 has been considered. P450 3A4 expression appears to be decreased as a result of liver cirrhosis or cancer [983, 1257, 1446]. P450 3A4 levels were also decreased in celiac disease and reversed by a change in diet [1447].

The interactions of herbal medicines with P450 3A4 have already been mentioned and are one of the worst problems with these mixtures [1448]. One of the most studied issues is St. John's wort, which induces P450 3A4 as an agonist of the receptor PXR [1449, 1450]. The induction of P450 3A4 by St. John's wort has been responsible for the loss of the effectiveness of oral contraceptives [83, 1451]. The resulting pregnancies are the result of more rapid elimination of 17α-ethinylestradiol, a phenomenon previously reported for P450 3A4 induction by rifampicin and barbiturates [26, 82, 90].

P450 3A4 is also of interest regarding cancer, regarding exogenous carcinogens (Fig. 9.10), drugs used to treat cancer, and metabolism of ste-

roids or other compounds that may affect cancer risk or response to chemotherapy. Some chemical carcinogens activated by P450 3A4 are shown in Table 9.8. The activation and detoxication of aflatoxin B_1 have already been discussed in the context of 3α-hydroxylation (to aflatoxin Q_1) and formation of the highly reactive *exo*-8,9-epoxide [29, 1331]. However, aflatoxin B_1 is a hepatocarcinogen and must reach the liver to cause damage. In a rat model, induction of rat P450 led to an increase in small intestinal DNA adducts, suggesting that activation of aflatoxin B_1 at this site constitutes a detoxication process, in that these cells are rapidly sloughed and do not progress to tumors [1452].

P450 3A4 genotypes have been reported to be related to leukemias caused by prior treatment with epipodophyllotoxin [1453]. P450 3A4 expression, measured at the mRNA level, has shown an inverse correlation with response of breast cancer patients to docetaxel, presumably due to changes in bioavailability [1454]. However, no relationships were found for any P450 3A4 genotypes in therapy-related myeloid malignancies [1455]. One of the more controversial issues involves whether P450 3A4 genotypes are linked with prostate cancer, with reports for and against an association [1456–1461]. The point should be made that strong evidence for a change in an accepted P450 3A4 phenotype has not been made in many of these cases.

9.7.21 P450 3A5

P450 3A5 has 85% sequence identity with P450 3A4 and, although generally accepted to have less importance than P450 3A4, is of interest because of its highly polymorphic and racial distribution and possible relevance to clinical issues with P450 3A subfamily reactions.

9.7.21.1 Sites of Expression

P450 3A5 ("HLp3") was first purified from human adult liver and found to be polymorphically expressed [1462]. Gonzalez used a liver sample apparently expressing only P450 3A5 and not 3A4 to clone a cDNA [1463].

P450 3A5 expression has been reported in liver, small intestine, kidney, lung, prostate, adrenal gland, and pituitary [1253, 1464–1466]. In human brain, both P450 3A4 and 3A5 were detected (by form-specific antibodies) in the microsomal fractions of cortex, hippocampus, basal ganglia, amygdala, and cerebellum [1252]. Both (P450 3A4 and 3A5) were localized in the soma and axonal hillock of neurons and varied according to cell type and cell layer. Some researchers have reported expression of P450 3A5 in peripheral blood cells (and *not* P450 3A4) [1467], but others have not [1253].

P450 3A5 is expressed in fetal liver, in contrast to P450 3A4, but in a polymorphic manner [1468]. The overall expression of P450 3A5 (mRNA) as a part of all subfamily 3A P450 transcripts has been estimated at 2% [1253]. However, only about 25% of Caucasians express P450 3A5, and when it is present, the level is usually less than that of P450 3A4. However, a few individuals have been identified in which P450 3A5 is the predominant P450 3A subfamily enzyme. The variability in expression levels has been linked to a polymorphism (*vide infra*).

Recently Achour et al. [55] have used LC–MS to quantitate P450 3A5 in human liver microsomes, with a mean of 14 pmol/mg microsomal protein (and a 100-fold range).

9.7.21.2 Regulation

The regulation of the *CYP3A5* gene seems to be similar to that of *CYP3A4,* although P450 3A5 does not seem as inducible. The fetal/adult selectivity of P450 3A4/3A7 is not seen [1468].

Maurel [1469] reported genomic clones and found a CATA box (not TATA) in the promoter. The responses to glucocorticoids are probably explained by the PXR system [1470]. The general conclusion has been reached that P450s 3A4 and 3A5 are coregulated in the liver and intestine, in terms of transcriptional control [1471], although other factors may alter the expression [1348].

9.7.21.3 Genetic Variation

At least 26 alleles have been identified, and six more SNVs have not been classified regarding haplotype yet (http://www.cypalleles.ki.se).

Most of the genetic variants are loss of function due to splicing, etc. However, one with a single coding change (*11, Y53C) results in a protein with only ~20% of the wild-type catalytic activity towards nifedipine [1472]. The *17 allele is also very deficient in activity [1313].

Individuals with the *1 allele express the functional (wild-type) protein, but those with the *3 allele express low to undetectable levels (of P450 3A5). The allele frequencies vary considerably with regard to race, with the frequency of the *1 allele being 10–30% in Caucasians, 30–40% in Asians, and 50–70% in an African American population [1310, 1473, 1474]. In *1 homozygotes, P450 3A5 levels as high on 50% of the total subfamily 3A P450 pool have been reported [1310]. If there is a difference in catalytic activity between P450 3A4 and 3A5, the genotype may be important (see Sect. 7.21.7, *vide infra*).

Other alleles are known, including changes in the 5′-regulatory region where transcription factors bind [1475].

The in vivo consequences of 3A5 polymorphism are not clear. For instance, Huang found no significant effect of the *3 polymorphism on midazolam pharmacokinetics [1476].

9.7.21.4 Substrates and Reactions

Since the discovery of P450 3A5, the catalytic selectivity has been known to be similar to that of P450 3A5 [1462], and subsequent comparisons with P450 3A4 confirmed this view [1477]. However, a general problem with P450 subfamily 3A P450 enzymes is that they are sensitive to the membrane environment. Many P450 3A4 and 3A5 reactions—but not all reactions—are stimulated by the addition of cytochrome b_5 [736, 1478]. Lee and Goldstein [1479] reported similar patterns of dependence for P450s 3A4 and 3A5. In a few cases, the selectivity of P450 3A5 for different oxidation sites appears to differ from that of P450 3A4, e.g., aflatoxin B_1 3α-hydroxylation versus 8,9-epoxidation [1406, 1478]. Wrighton reported an extensive comparison of many reactions by recombinant P450s 3A4, 3A5, 3A7 under identical reconstitution conditions and concluded that P450 3A5 had equal or reduced activity compared to P450 3A4 in all cases examined [1316].

One of the most important issues is to what extent P450 3A5 participates in a reaction, relative to P450 3A4. If P450 3A5 plays a major role, then P450 3A5 genetic variations (Sect. 7.21.3) may become important in vivo. Niwa et al. [1375] have catalogued a number of reactions and found that the ratio of P450 3A4/3A5 activity varies. Amlodipine oxidation is catalyzed almost exclusively by P450 3A4 [1480].

Interesting recent results indicate that P450 3A5 is more active than P450 3A4 in some reactions. One is the activation of lapatinib [1481]. Another is the O^6-demethylation of thebaine (Fig. 9.17), a critical step in the synthesis of endogenous morphine [906], where P450 3A5 is >10-fold more active than P450 3A4 [906]. The presence of P450 3A5 in brain (Sect. 7.21.1) may have implications in the relevance of the pathway.

Another issue with P450 3A4 (and some other P450s) is cooperativity (Sect. 7.20.4, *vide supra*). Niwa et al. [1375] have reviewed heterotropic cooperativity in P450s, including human subfamily P450 3A P450s. Recently, P450 3A5 has been shown to exhibit homotropic cooperativity in the oxidation of thalidomide [1360, 1482].

9.7.21.5 Structure

Because of the similarity of reactions of P450s 3A4 and 3A5, homology models based on P450 3A4 structures are probably reasonably valid for P450 3A5. Relatively little site-directed mutagenesis of P450 3A5 has been done, but one study of note is the effort by Correia and Halpert to utilize the differences in reactions with aflatoxin B_1 [1331, 1478] to probe the effects of changing residues in the active site [1406].

9.7.21.6 Inhibitors

In general, the P450 3A4 inhibitors also inhibit P450 3A5. For instance, ketoconazole and fluconazole inhibit both P450s 3A4 and 3A5 [1483]. The mechanism-based inactivator gestodene [1250] also inhibits P450 3A5 [1477].

In light of the importance of distinguishing whether reactions are catalyzed by P450 3A4 or 3A5 (*vide supra*), Li et al. [1432] have described a P450 3A4-selective inhibitor (1-(4-imidazopyridinyl-7-phenyl)-3-(4′-cyanobiphenylurea (SR9186))) that can be utilized for this purpose. Another selective P450 3A4 inhibitor (not affecting P450 3A5) is CYP3cide (PF-4981517; 1-methyl-3-[1-methyl-5-(4-methylphenyl)-1*H*-pyrazol-4yl]-4-[13*S*]-3-piperidin-1-yl-pyrrolidin-1-yl]-1*H*-pyrazolo[3,4-*d*] pyrimidine) [1430], a mechanism-based inactivator. Another is ML-368 [1431].

Cannabidiol, a major substituent of marijuana, inhibits several human P450s and inhibited P450 3A5 with an IC_{50} tenfold lower than P450 3A4 [939].

9.7.21.7 Clinical Issues

At this point, the significance of the wide variability in P450 3A5 is still difficult to assess. As mentioned previously, Huang [1476] found no significant effect of the *3 allele on midazolam pharmacokinetics in Chinese individuals. However, it is possible that the extrahepatic expression [1253] may influence the course of particular drugs and other chemicals.

More recent studies have reported a lack of a major contribution in the cases of oxidation of amlodipine [1480], midazolam [1484], and atorvastatin [1485]. There has been considerable interest in whether P450 3A5 genetic testing is of use in dosing of tacrolimus (FK-506), an immunosuppressant widely used in organ transplantation, long known to involve P450 3A4 oxidation [1486, 1487]. P450 3A5 genotyping (with tacrolimus use) has been concluded by some to be beneficial [1488, 1489] but not by others [1490, 1491].

9.7.22 P450 3A7

Early work in the field of human P450 research by Kamataki and his associates with fetal samples led to the purification of a P450 termed HFLα, now known as P450 3A7 [8, 24]. Early research established that this is a major P450 in fetal liver (not in adult liver) and that the enzyme could catalyze several reactions [24].

9.7.22.1 Sites of Expression

Early work established that P450 3A7 is the major P450 present in fetal liver [24] and is also present in other fetal tissues, including kidney, adrenal, and lung [1492]. Further work by Kamataki's group showed the existence of some immunochemically detectable P450 3A7 in gynecologic tumors and in human placenta, but interestingly not in cynemologous monkey placenta [1493]. Guzelian's group also reported P450 3A7 protein in human placenta and endometrium, with elevation in the latter site during pregnancy or during the secretory phase of the menstrual cycle [1494]. Subsequently Sarkar et al. [1495] reported tenfold greater expression of P450 3A7 in endometrium in the proliferative rather than the secretory phase. Hakkola et al. [1496] reported some expression of P450 3A7 mRNA in some first trimester placenta but not in full-term placenta [381]. Juchau's group found expression of P450 3A7 in early fetal tissue (50–60 days) [1497]. Schuetz et al. [1498] found P450 3A7 mRNA in all fetal liver samples analyzed and also reported its presence in one half of adult liver samples. However, the issue may be the level of expression, because Kamataki's group [174] had reported the fetal > adult selectivity. de Wildt et al. [1260] also found fetal specificity and only very low levels of P450 3A7 in adults. P450 3A7 expression was high during embryonic and fetal life and decreased rapidly during the first week of life. Similar findings were reported by Hakkola et al. [1468]. Also, the variability of P450 3A7 expression was fivefold in fetal tissue (and 77-fold in mRNA). In another report [1499], P450 3A7 also disappeared rapidly after infancy.

More recently, Gonzalez and his associates [1500] have developed a mouse model in which P450 3A7 is expressed in the fetus and a decrease is seen after birth. In the model, the CYP3A7 is regulated by glucocorticoids through the glucocorticoid receptor.

9.7.22.2 Regulation

The regulation of this gene is complex, as one might expect after considering the temporal patterns of expression during development (*vide supra*). Kamataki's group published the cDNA [1501] and genomic [1502] sequences, which are similar to those of P450 3A4. However, more identity (~90%) is seen in the coding region than elsewhere [1469, 1502]. Recent work by Koch et al. [1253] reestablished that P450 3A7 only accounted for <2% of all P450 expression in adult human liver; a bimodality of P450 3A7 expression was seen, however. P450 3A4 and 3A7 constructs were expressed in various cell lines by Ourlin et al. [1282], who showed differential responses to C/EBPα and DBP. As in the case with P450 3A4, P450 3A7 was inducible by rifampicin in cell culture [1503]. P450 3A7 has a functional PXR element [1504], as does P450 3A4 (*vide supra*), explaining the rifampicin response. Thus, one would expect fetal P450 3A7 induction by the usual P450 3A4 inducers.

Bertilsson et al. [1505] reported a distal XREM in the *CYP3A7* gene. An NFκB-like element in *CYP3A7* is inactive in *CYP3A4* [1506], and this element has recently been shown to respond to p53. *CYP3A7* expression is regulated by Sp1, Sp3, HNF-3β, and USF1. Far upstream (~11 kb), there are HNF-1 and HNF-4 and USF1 elements, which differ from the *CYP3A4* gene. Exactly how this and other sequence differences are involved in the rapid onset of P450 3A4 and decrease in P450 3A7 shortly after birth [175] is unclear.

The exact basis of the postpartum shift from P450 3A7 to 3A4 expression is still not clear. Although P450 3A7 has a PXR element, Matsunuga et al. [1507] reported that P450 3A7 was induced by dexamethasone but not rifampicin in fetal human hepatocyte culture. This finding is consistent with the report of Pang et al. [1500] with the transgenic P450 3A7 mouse model, in which induction with glucocorticoids suggests control by the glucocorticoid receptor, not PXR, is important [1473].

The *CYP3A7*1C* alleles is expressed in adult liver because the G>219T substitution creates a binding site for HNF-3 and the associated A233C change destroys an HNF-3 binding site, creating a putative octamer identical to that found upstream for P450 3A4 [1473, 1508].

Phosphorylation of P450 3A7 has also been detected in vivo [297].

9.7.22.3 Genetic Variation

At least seven alleles are known (http://www.cypalleles.ki.se). One (*1C) has been mentioned above regarding its expression in adults [1473, 1509]. A null allele (frameshift) (*3) has been identified [1510]. The *1C allele was associated with a 50% reduction in serum DHEA 16α-sulfate (in adults) [1511]. The effect of this in fetal life is unknown. Some interesting variants of *CYP3A7* genes have been reported. An mRNA species was found that contains exons 2 and 13 of a nearby *CYP3A* pseudogene spliced at the three end [1512]. The *CYP3A7*1C* allele is unusual in the sense that a part of the *CYP3A4* promoter replaces the corresponding region of *CYP3A7* (ER6 motif) and thus confers high levels of expression to *CYP3A7*1C* [1513].

The overall variability of P450 3A7 mRNA in fetal liver varied 630-fold [1514]. This observed variability could not be attributed to the *2 or other known polymorphisms.

9.7.22.4 Substrates and Reactions

Early studies with P450 3A7 purified from fetal liver established that testosterone 6β-hydroxylation is catalyzed by this enzyme [1515]. Another early study indicated 16α-hydroxylation of DHEA 3-sulfate [1516]. These activities were later verified with the use of recombinant P450 3A7 [1517].

In general, P450 3A7 has catalytic activities rather similar to P450 3A4 and 3A5 [1518, 1519]. Activation of aflatoxin B_1 [1520–1522] and heterocyclic amines [1520] has been observed in various recombinant and transgenic systems, including transgenic mice [1523]. Retinoic acid 4-hydroxylation by P450 3A7 has also been reported [1524]. Wrighton's laboratory reported an extensive comparison of catalytic activities and concluded that rates for P450 3A7 are generally considerably lower for P450 3A7 than for P450 3A4 or 3A5 under similar conditions [1316].

The consensus about generally similar but quantitatively lower catalytic activities of P450

3A7 relative to P450s 3A4 and 3A5 still appears to hold [1473], although some new information is available. Lee et al. [1525] reported that P450 3A7 uniquely had a similar level of estrone 16α-hydroxylation activity compared to 2-hydroxylation, in contrast to P450 3A4. That was not the case for 17β-estradiol. The possibility exists that P450 3A7 may be important in the local or systemic formation of 16α-hydroxyestrone (which is procarcinogenic in some animal models).

Two of the substrates of P450 3A7 that may be most important are DHEA 3-sulfate (16α-hydroxylation) and retinoic acid, in terms of protection of the fetus. However, the finding that fetal levels of P450 3A7 can vary 630-fold [1514] raises questions about how important such any regulation of these steroid and retinoid levels by P450 3A7 really is.

9.7.22.5 Structure

Much less has been done with P450 3A7 than with P450s 3A4 and 3A5. Because the catalytic selectivity of P450 3A7 is similar to P450s 3A4 and 3A5, models are probably about as applicable. One point of interest is the work of Kamataki's group showing that the substitution T485P improved holoprotein expression in *E. coli* [1526].

9.7.22.6 Inhibitors

Inhibitors have not been studied extensively, but presumably most general inhibitors of P450 3A4 are effective with P450 3A7, e.g., ketoconazole, TAO, etc.

9.7.22.7 Clinical Issues

The general point has already been made that P450 3A7 is the major human fetal P450 and therefore makes a major contribution to drug metabolism in the fetus. Thus, many, if not most, of the considerations regarding drug interactions etc. with P450 3A4 should be considered with respect to the fetus during pregnancy. At this time, there is still no clear consensus that the level or activity of P450 3A7 in the fetus will have a major physiological effect due to altered metabolism of endogenous compounds. The best candidates, if indeed these are candidates, are retinoids and DHEA 3-sulfate. What is probably of more concern is the role of P450 3A7 in the (fetal) metabolism of drugs. Even in pediatric medicine, there is limited information to guide dosing [1527, 1528] and the knowledge base regarding in vivo fetal drug metabolism is even more limited.

Another potentially important aspect is a report that P450 3A7 expression increases in hepatocellular carcinoma [1529], possibly as a part of dedifferentiation.

9.7.23 P450 3A43

9.7.23.1 Sites of Expression

In 2001, three groups reported the characterization of a fourth subfamily 3A P450 member, P450 3A43 [1530–1532]. The sequence identity with other P450 3A subfamily members is 71–76%. Expression (mRNA) is seen in liver, kidney, pancreas, and prostate, and testis [1473]. More recently, high levels of expression have been reported in brain, as high or higher than in liver [1254]. The results are discordant, in that previously the 3A43/3A4 mRNA expression ratio was $1/10^3$, but in the brain study the liver ratio was one fifth [1254]. Very low levels (<1 pmol/mg protein) have been detected in human liver microsomes using LC–MS [54, 55].

9.7.23.2 Regulation

As with other P450 3A subfamily members, rifampicin induces P450 3A43 [1530], presumably via the PXR system.

9.7.23.3 Genetic Variation

Genetic polymorphism in the *CYP3A43* gene has been reported [1533], and the http://www.cypalleles.ki.se website currently lists five alleles. Two are frameshifts and should not yield functional protein.

9.7.23.4 Substrates and Reactions

The initial studies with heterologously expressed P450 3A43 (in *E. coli*) showed only low testosterone 6β-hydroxylation activity (a marker for other 3A subfamily members) [1531]. Agarwal et al. [1254] reported different catalytic specificity in alprazolam oxidation compared to P450 3A4 and a relatively high level of activity, con-

cluding that P450 3A43 was more important than P450 3A4 in brain metabolism of this drug.

9.7.23.5 Structure

No structures or homology models of P450 3A43 have been reported.

9.7.23.6 Inhibitors

Specific P450 3A43 inhibitors have not been reported, perhaps in part due to the low catalytic activities. Presumably, other P450 3A subfamily inhibitors such as ketoconazole would be effective.

9.7.23.7 Clinical Issues

A polymorphism in P450 3A43 has been used to explain a racial difference in olanzapine clearance [1534]. The conclusion is surprising in that another study reported that P450s 1A2 and 2D6 (and FMO) are more involved in olanzapine metabolism [1535].

9.7.24 P450 4A11

9.7.24.1 Sites of Expression

P450 4A11 was first cloned from a kidney cDNA library [1536] and later identified in human liver microsomes [1537]. The originally reported P450 4A11 sequence was subsequently found to be that of P450 4A22 [1538] and the correction has been made. P450 4A11 is expressed largely in the liver and kidney. A proteomic study found P450 4A11 peptides in all human livers sampled [635]. The level of expression of P450 4A11 is much higher than that of P450 4A22 [1539].

9.7.24.2 Regulation

P450 4A11 is induced in HepG2 cells by both peroxisome proliferators (PPARα) and dexamethasone [1539]. Presumably a PPARα site(s) exists in the gene. Clofibrate is also an inducer [1540].

9.7.24.3 Genetic Variation

In addition to wild-type P450 4A11, at least nine variants are known (http://www.cypalleles.ki.se) [1541]. As discussed later, there is considerable interest in the variants in relationship to hypertension and other cardiovascular diseases [1542].

9.7.24.4 Substrates and Reactions

P450 4A11 catalyzes the ω-hydroxylation of fatty acids, with a small amount of ω-1 product [1536, 1537, 1543–1545]. 20-Hydroxyeicosatetraenoic acid (20-HETE), a primary product, is a potent vasoactive and natriuretic eicosanoid in human kidney, and there is considerable interest in the P450 4A11-catalyzed conversion of arachidonic acid to this product (Sect. 7.24.7, Clinical Issues, *vide infra*). Some prostaglandins (stable analogs) have also been reported to be ω-hydroxylated by P450 4A11, including 9,11-diazo-15-deoxy-PGH_2 (U51605), 9,11-epoxymethano-PGH_2 (U44069), and 11,9-epoxymethoPGH$_2$ (U46619). Thus, P450 4A11 may oxidize other long-chain alkyl molecules.

Some other points should be made about these reactions of P450 4A11. First, in P450 4A11 much of the heme is covalently linked to the apoprotein [1546]. However, this attachment is not critical to catalytic activity [1546]. Second, the reaction is stimulated ~twofold by cytochrome b_5 [1547], and the stimulation does not occur without the heme [1548], arguing for electron transfer. Kinetic analysis indicates that the "second" electron transfer (from cytochrome b_5) and the C–H bond-breaking step are both rate limiting [1548]. An apparently high intrinsic kinetic deuterium isotope effect shows considerable attenuation. Finally, several of the sulfhydryls (cysteines) in the protein are readily oxidized (to a disulfide and to sulfenic acids), and the reactions are enhanced by reductants, e.g., dithiothreitol, tris(2-carboxyethyl)phosphine, glutathione. The latter phenomenon is observed in human liver microsomes, but its in vivo relevance is still under investigation [1549].

9.7.24.5 Structure

No crystal structures of P450 4A11 have been reported. Some homology models have been published [1550, 1551]. As mentioned earlier, two phenomena observed with P450 4A11 are the dependence of catalytic activity on free thiols [1549] and the autocatalytic covalent attachment of heme [1546].

9.7.24.6 Inhibitors

HET0016 is a strong competitive inhibitor of P450 4A11 and can be used in vivo (in animals) [1552]. Another inhibitor is "20-SOLA" (2,5,8,11,14,17-hexaoxanonadecan-19-yl 20-hydroxyeicosa-6(Z),15(Z)-dienoate).

9.7.24.7 Clinical Issues

P450 4A11 does not appear to be involved in the metabolism of any drugs, and the major issue is the role of P450 4A11 in cardiovascular diseases, particularly salt-sensitive hypertension [1542, 1553–1571].

The hypertension problem is complex. An association between the *rs1126742* C allele (coding for an F434S variant) and hypertension was reported in 2005 [1542] and has been rather reproducible in other human studies, with some exceptions [1572]. The working hypothesis has been that the 20-hydroxylation (ω) of arachidonic acid is involved, in that this is the only measured physiologically relevant catalytic activity. The F434S variant had a catalytic efficiency ~40% lower than the WT (*1) enzyme [1542]. Deleting the *Cyp4a10* or *Cyp4a14* gene from mice renders them hypertensive, but neither of these enzymes is an effective arachidonic ω-hydroxylation catalyst [1573, 1574]. P450 4a12 is the major arachidonate ω-hydroxylase in mice [1575], but this gene has not been deleted yet. It is possible that the 20-HETE produced by P450 4A11 induces P450 2C/c subfamily enzymes that make protective epoxides, and deletion of mouse *Cyp2c44* also causes hypertension [1576]. However, transgenic mice expressing human P450 4A11 have higher levels of plasma 20-HETE and have hypertension [1577]. Thus, it is not clear exactly what role P450 4A11 has in hypertension. Unresolved issues are the importance of the site of P450 4A11 expression within the kidney, in that 20-HETE can act as a vasoconstrictor or a vasodilator, and the effect of the *rs1126742* genotype on the stability and level of expression of P450 4A11 in the kidney and liver.

9.7.25 P450 4A22

Relatively little is known about P450 4A22. The originally reported *CYP4A11* gene [1578] was subsequently shown to be *CYP4A22* [1538], but the cDNA and protein have not been reported. The similarity of the two genes is 96%.

Johnson's laboratory [1539] has reported that P450 4A22 is expressed at lower levels than P450 4A11 in human liver, as well as kidney [1538]. There was no correlation of expression levels of P450 4A11 and 4A22 in human liver [1539]. P450 4A22 expression could not be observed in HepG2 cell or PPARα-overexpressing cells [1539].

P450 4A22 protein has been detected in human liver using LC–MS [635].

9.7.25.1 Regulation

Relatively little information is available. Savas et al. [1539] reported that P450 4A22 was only expressed at low levels in human hepatoma HepG2 cells and was refractory to treatment with the PPARα inducer Wyeth 14,643 or dexamethasone, in contrast to P450 4A11.

9.7.25.2 Genetic Variation

The *CYP4A22* gene is highly polymorphic [1551, 1579]. At least 22 variants have been identified (http://www.cypalleles.ki.se).

9.7.25.3 Substrates and Reactions

Presumably the catalytic activity of the enzyme is fatty acid ω-hydroxylation. However, a literature search did not reveal an actual assay with P450 4A22, and it is not known if the protein has ever been expressed.

9.7.25.4 Structure

No structure is available, but at least two homology models have been published [1551, 1580].

9.7.25.5 Inhibitors

No inhibitors have been reported, although HET0016 might be expected to be an inhibitor in light of its activity against P450 4A11.

9.7.25.6 Clinical Issues

In contrast to P450 4A11, there are no clinical issues with P450 4A22 due to the evidence for lower levels of expression.

9.7.26 P450 4B1

9.7.26.1 Sites of Expression

P450 4B1 was cloned by Nhamburo et al. [1581] from a human lung cDNA library. P450 4B1 expression has been reported (in addition to lung) in kidney, bladder [1582], breast [1583], and prostate [1584]. Expression has also been reported in bladder and breast tumors [1582] and lung tumors [1585]. It should be emphasized that the tissue-selective expression of P450 4B1 varies considerably among species, as discussed by Baer and Rettie [1586].

9.7.26.2 Regulation

As with the tissue-specific expression (*vide supra*), the regulation of P450 4B1 expression varies considerably among species [1586], and caution is advised in the extrapolation of results from any animal species to humans. Poch et al. [1587] utilized A549 lung carcinoma cells and HepG2 human hepatocarcinoma cells to identify a proximal positive regulatory element located between −118 and −73, a liver-selective negative regulatory element located between −457 and −216, and a distal lung-selective positive element located between −1052 and −1008. Three possible binding sites were found for the Sp/XKLF family of transcription factors. The Sp1 and Sp3 transcription factors regulate P450 4B1 through the proximal regulatory element, but the transcription factors involved in the distal lung-selective positive element could not be identified.

9.7.26.3 Genetic Variation

Genetic variation in the *CYP4B1* gene appears to be extensive, with several of the variants leading to loss of function [1588, 1589]. The interest in P450 4B1 variation has been linked to possible roles in cancers [1589].

9.7.26.4 Substrates and Reactions

The literature contains a considerable amount of information concerning substrates and reactions of P450 4B1 enzymes. However, most of this involves animal systems, and Baer and Rettie [1586] have pointed out the problems in extrapolation to human P450 4B1. A number of technical problems have plagued heterologous expression studies with human P450 4B1 [1586], and accordingly the information is limited. A transgenic mouse model expressing human 4B1 in (mouse) liver was developed sometime ago [1590]. More recently, a *CYP4B1* knockout mouse has also been developed [1591], and it should be possible to combine these systems.

Substrates and reactions have been summarized by Baer and Rettie [1586]. Proven substrates are lauric acid and 2-aminofluorene, although both of these must only be considered models for related compounds of interest.

9.7.26.5 Structure

No structure is available. Heme is covalently linked to the apoprotein (Glu-310) through an ester linkage [1592]. Apparently no homology models have been published, and the issues about species extrapolation etc. (*vide supra*) would suggest caution in such efforts.

9.7.26.6 Inhibitors

No specific inhibitors have been identified.

9.7.26.7 Clinical Issues

There are two clinical issues regarding P450 4B1. One is a possible epidemiological link to cancers [1582, 1589], largely driven by work with P450 4B1 from animal models. The other issue is the potential use of P450 4B1 (endogenous or instilled by gene therapy) in the bioactivation of cancer prodrugs [1593].

9.7.27 P450 4F2

9.7.27.1 Sites of Expression

The Kusenose laboratory reported the cloning of a human liver cDNA corresponding to the leukotriene B_4 ω-hydroxylase [1594]. The site of expression was distinct from P450 4F3, which is restricted to polymorphonuclear leukocytes. P450 4F2 is found not only in liver but also in several extrahepatic tissues [1595], including kidney (S2 and S3 segments of proximal tubules, in cortex, and outer medulla). The extent of variation of P450 4F2 in human liver was ~fivefold [1596].

9.7.27.2 Regulation

Relatively limited information about regulation is available about P450 4F2. Expression is controlled by sterol regulatory element-binding proteins (SREBPs) [1597]. In human hepatocytes, lovastatin induced P450 4F2 and this effect was blocked by 25-hydroxycholesterol.

9.7.27.3 Genetic Variation

At least two genetic variants are known (http://www.cypalleles.ki.se), coding for the W12G and V433M forms. The latter has been studied in detail with regard to its role in warfarin metabolism, i.e., association with increased warfarin dosing [1598, 1599]. The genotype controls the level of protein expression [1600].

9.7.27.4 Substrates and Reactions

P450 4F2 catalyzes ω-hydroxylation of several lipids, including leukotriene B_4 [1596, 1601], arachidonic and [1545], and 6-*trans*-leukotriene B_4, lipoxin A_4, 8-hydroxyeicosatetraenoic acid, 12-hydroxyeicosatetraenoic acid, and 12-hydroxystearic acid [1602]. The physiological relevance of some of these reactions is of interest, but the effects of variability of P450 4F2 have not been demonstrated. Part of the interest lies in the fact that leukotriene B_4 is a potent proinflammatory agent [1595, 1596].

In addition, P450 4F2 can also catalyze ω-hydroxylation of arachidonic acid [1598].

Several drugs are also oxidized by P450 4F2, including DB289 (2,5-bis[4-amidinophenyl] furan-*bis*-*O*-methylamidoxime) [1603] and fingolimod (FTY720) [1604]. A polymorphism (V433M) in P450 4F2 has been shown to affect the clinical warfarin dose [1599], but the enzyme does not oxidize warfarin. Rettie and his associates showed that the reason was that P450 4F2 is a vitamin K oxidase, explaining the effect in terms of a physiological reaction [1605]. The reaction involves ω-oxidation and further oxidation to the carboxylic acid [1600]. P450 4F2 is also an ω-oxidase for vitamin E [1606].

9.7.27.5 Structure

No structural information is available yet for P450 4F2.

9.7.27.6 Inhibitors

No inhibitors have been reported. In that this is an ω-hydroxylase, HET0016 might be expected to inhibit.

9.7.27.7 Clinical Issues

The major clinical issue with P450 4F2 is the role in warfarin dose adjustment [1607–1610] due to its activity in vitamin K oxidation [1605]. The issue is not a change in the activity of the enzyme (V433M) but the protein stability [1600].

9.7.28 P450 4F3

9.7.28.1 Sites of Expression

A P450 4F3 cDNA was first cloned from a human leukocyte library in 1993 [1611]. The *CYP4F* gene family is clustered in the p13 region of chromosome 19, and P450 4F3 expression results in the synthesis of two enzymes, P450 4F3A and P450 4F3B, resulting from alternate splicing of a single pre-mRNA precursor [1612]. As a result of tissue control, P450 4F3A contains exon 4 (but not 3) and is expressed in neutrophils. P450 4F3B contains exon 3 (but not 4) and is expressed in fetal and adult liver and kidney, trachea, and gastrointestinal tract [1613, 1614].

9.7.28.2 Regulation

The tissue-specific expression of P450 4F3A/B has been mentioned (*vide supra*). Induction of transcription has been reported with prostaglandin A1 (4F3B) in a human hepatocyte-derived cell line [1615] and with benzene metabolites (in promyelocytic leukemia cell lines and in blood neutrophils [1616, 1617]). P450 4F3B expression is associated with differentiation of HepaRG human hepatocytes and unaffected by fatty acid overload [1618]. Statins have been reported to increase P450 4F3 in human liver cells through a PXR-dependent mechanism [1619]. All-*trans*-retinoic acid has been reported to induce P450 4F3A in HL-60 cells [1620].

9.7.28.3 Genetic Variation

Although the *CYP4F3* gene is subject to alternate splicing, few reports of genetic variation have yet

appeared. An SNV has been related to celiac and Crohn's disease [1612, 1621].

9.7.28.4 Substrates and Reactions

The two proteins generated by alternate splicing, P450 4F3A and 4F3B, have different catalytic specificities. P450 4F3 is an ω-hydroxylase, but the 4F3A form is more active with leukotriene B_4 and the 4F3B form is more efficient with arachidonic acid [1614].

P450 4F3B has reported to have some ability to oxidize the drug fingolimod (FTY720) [1604].

9.7.28.5 Structure

Little is known about the active site, including the features associated with the differential selectivity of the P450 4F3A and 4F3B enzymes. A fraction of the heme was shown to be covalently attached to the protein [1622].

9.7.28.6 Inhibitors

A search did not identify reports of inhibitors of P450 4F3A/B. It is possible that HET0016 might be one, on the basis of its inhibition of other ω-hydroxylases.

9.7.28.7 Clinical Issues

As mentioned under Sect. 7.28.3 (*vide supra*), there has been some association of SNVs with celiac and Crohn's disease [1612, 1621].

9.7.29 P450 4F8

9.7.29.1 Sites of Expression

Bylund et al. [1134] first isolated the cDNA from a human seminal vesicle library. Expression has also been reported in human epidermis, hair follicles, sweat glands, corneal epithelium, proximal renal tubules, and epithelial linings of the gut and urinary tract [1623].

9.7.29.2 Regulation

P450 4F8 expression is upregulated in psoriasis [1623, 1624]. The mechanism has not been elucidated. A possible relationship with fenofibrate treatment has been reported [1625].

9.7.29.3 Genetic Variation

No reports on genetic variation were identified in a search.

9.7.29.4 Substrates and Reactions

P450 4F8 was shown to catalyze hydroxylation of prostaglandin endoperoxides [1134, 1626]. The recombinant enzyme also catalyzed the ω-2 hydroxylation of arachidonic acid and three stable prostaglandin H_2 analogs, but prostaglandins D_2, E_1, E_2, and $F_2\alpha$ and leukotriene B_4 were poor substrates [1626]. These findings are of relevance in that 19-hydroxyprostaglandins have several biological activities [1623]. (19*R*)-Hydroxy prostaglandins E_1 and E_2 are the main prostaglandins of human seminal fluid. Bylund et al. [1626] have proposed that ω-1 hydroxylation of prostaglandins H_1 and H_2 by P450 4F8 occurs in seminal vesicles and that isomerization to (19*R*)-hydroxyprostaglandin E is the result of the action of prostaglandin E synthase.

P450 4F8 has also been reported to form ω-3 hydroxy products of arachidonyl epoxy alcohols with a 11,12-epoxy-10-hydroxy configuration [1627]. The 8,9- and 11,12-epoxides are also substrates for ω-3 hydroxylation.

9.7.29.5 Structures

No information is available about the structure or active site.

9.7.29.6 Inhibitors

No inhibitors have been reported for P450 4F8.

9.7.29.7 Clinical Issues

As mentioned (*vide supra*), P450 4F8 expression is associated with psoriasis [1623, 1624], but its role in the etiology of the disease is unclear. P450 4F8 has also been identified as a potential target in prostate cancer [1628].

9.7.30 P450 4F11

9.7.30.1 Sites of Expression

P450 4F11 has been reported to be expressed (at the mRNA level) mainly in liver, followed by

kidney, heart, skeletal muscle, and brain [1629]. Expression of P450 4F11 in liver has been confirmed at the protein level [297, 635, 1600].

9.7.30.2 Regulation

The regulation of P450 4F11 has been studied in cell culture. In human keratinocyte (HaCaT) cells, the proinflammatory cytokines TNFα and interleukin-1β induce P450 4F11 transcription. The c-Jun N-terminal kinase (JNK) pathway is involved [1630]. An RXR agonist induced P450 4F11 transcription and a retinoic acid receptor (RAR) agonist attenuated transcription [1630].

In HepG2 cells (human liver carcinoma line), TNFα also stimulated P450 4F11 transcription through the JNK pathway, and NFκB attenuated transcription [1631].

9.7.30.3 Genetic Variation

Only limited genetic variation has been reported in P450 4F11. The *rs11553651* (15016G>T) variant was reported not to be associated with breast cancer in a study of Mexican women [1230]. A D466N substitution is also known but did not influence vitamin K ω-hydroxylation [1600].

9.7.30.4 Substrates and Reactions

P450 4F11 catalyzes a number of reactions. In light of the fact that it has ~80% sequence identity to other subfamily 4F P450s, known to be ω-hydroxylases, it is not surprising that these reactions occur with P450 4F11 [1632]. Studies with P450 4F11 expressed in yeast, insect cells, and bacteria have all shown ω-hydroxylation activities towards several long-chain fatty acids, plus leukotriene B_4, lipoxin A_4, and 8-HETE (but not 5- or 12-HETE) [1632–1634]. Interestingly, much higher catalytic activity was seen with β-hydroxy fatty acids [1633, 1634]. The activities towards fatty acids are probably relevant in that (1) antibodies blocked activity in liver microsomes [1633], and (2) screening of liver extracts with recombinant P450 4F11 in a metabolomics approach also yielded stearic, oleic, arachidonic, and docosahexaenoic acids as substrates [1634]. In all of these cases, only ω-hydroxylation was observed.

Recently P450 4F11 has also been shown to be a catalyst of ω-hydroxylation of MK4, a menaquinone form of vitamin K [1600]. Further research has also shown a role in vitamin E oxidation [1635].

Some drugs are oxidized (at rates of $<1\ min^{-1}$) by P450 4F11, including amitriptyline, benzphetamine, chlorpromazine, erythromycin, ethylmorphine, fluoxetine, imipramine, pirenzepine, theophylline, and verapamil [1632, 1634].

9.7.30.5 Structure

No crystal structures are available. A homology model (based on P450s 2C5, 101A1, 102A1, 108A1, and 107A1) has been published [1632].

9.7.30.6 Inhibitors

No information on specific inhibitors of P450 4F11 has been published.

9.7.30.7 Clinical Issues

The extent to which P450 4F11 contributes to the oxidation of drug substrates is unknown. The same applies to vitamin K (MK4), and P450 4F2 is also a catalyst, with similar expression levels and catalytic efficiency [1600].

9.7.31 P450 4F12

9.7.31.1 Sites of Expression

P450 4F12 was originally cloned from human liver [1636] and small intestine [1637] cDNA libraries. Expression has been reported in liver, kidney, colon, small intestine, and heart [1636, 1638]. There are also reports of expression in gastrointestinal and urogenital epithelia [1639].

9.7.31.2 Regulation

PXR has been reported to regulate P450 4F12 expression in hepatocytes [1640].

9.7.31.3 Genetic Variation

At least seven variants in the *CYP4F12* gene have been identified, some with loss of function [1533, 1641]. Some of the activity changes have been reported with coding region variations [1642].

9.7.31.4 Substrates and Reactions

Reactions identified include ω-, ω-2, and ω-3 hydroxylation of arachidonic acid [1637] and the ω-hydroxylation of leukotriene B_4 [1636, 1637] and some prostaglandin analogs [1636]. Hydroxylation of the antihistamine ebastine has also been reported [1149].

9.7.31.5 Structure

No structures have been reported. The effects of variations at Tyr-125 have been reported [1642]. This enzyme has covalently bound heme, attached via Glu-328. Mutation at that site shifted the regioselectivity of oxidation of arachidonic acid [1642].

9.7.31.6 Inhibitors

Inhibitors of P450 4F12 have not been reported.

9.7.31.7 Clinical Issues

No clinical issues have been reported.

9.7.32 P450 4F22

9.7.32.1 Sites of Expression

P450 4F22 is associated with a skin disease called ichthyosis and accordingly is expressed in skin [1643]. Specifically, it is expressed at the onset of keratinization during skin development [1644]. Interestingly, an extensive analysis of (any) other sites of localization has not been reported.

9.7.32.2 Regulation

Although P450 4F22 is expressed during skin keratinization, molecular mechanisms of regulation have not been reported.

9.7.32.3 Genetic Variation

The ichthyosis is an autosomal recessive congenital disease, and several *CYP4F22* variants have been identified in individuals with the disease [1643, 1645–1647]: F59I, R243H, R372W, H456Y, and H436D, plus a frameshift and a large deletion.

9.7.32.4 Substrates and Reactions

Epoxy alcohols (HEETs) and epoxides (EETs) of arachidonic acid appear to be important for the water permeability barrier of skin (i.e., keeping it from drying out). P450 4F22 oxygenated arachidonic acid at the ω-2 position but did not oxidize HEETs [1627]. However, it has been pointed out that the reported catalytic activity is one to two orders of magnitude lower than that of P450 4F8, so the significance is unclear [1643].

The suggestion has been made that hepoxilins are more relevant lipids regarding this disease [1648]. The hepoxilins (or trioxilins) might be oxidized and play a role in signaling, rather than acting directly as barrier lipids [1643].

9.7.32.5 Structure

No structure is available but a homology model has been published [1649].

9.7.32.6 Inhibitors

No inhibitors have been reported, which is not surprising in terms of the limited evidence of a relevant reaction.

9.7.32.7 Clinical Issues

The clinical issue is ichthyosis, a serious disease. Variants (*vide supra*) in *CYP4A22* and several other genes [1650] are clearly involved. More remains to be learned about the molecular basis of the disease before intervention is possible.

9.7.33 P450 4V2

9.7.33.1 Sites of Expression

P450 4V2 is expressed at the mRNA level in a variety of tissues [1651]. The protein has been detected by LC–MS in (female) livers, although whether or not a gender difference really exists is unknown [635]. Antibodies have been used to detect P450 4V2 protein in the retina and corneum (eye), which is the site of most relevance [1652].

9.7.33.2 Regulation

The regulation of the P450 4V2 expression has not been studied, probably because of the emphasis on genetic variation as the major factor in P450 4V2 activity and disease relevance (*vide infra*).

9.7.33.3 Genetic Variation

P450 4V2 first attracted attention because a genetic defect was implicated in Bietti's crystalline dystrophy, a recessive degenerative eye disease [1651]. The information is not included on the http://www.cypalleles.ki.se website, but >80% of the mutant alleles related to the disease are attributed to three variants—two splice site alterations and one missense mutation (992 C>A, yielding the protein variant H331P) [1652]. The H331P was not expressed in HepG2 transfected with the cDNA and is concluded to be unstable [1652]. An I111T mutation has also been reported to cause the disease [1653].

9.7.33.4 Substrates and Reactions

P450 4V2 has been characterized as a fatty acid ω-hydroxylase [1654]. Subsequent work suggests ω-3 polyunsaturated as the substrates most relevant to Bietti's crystalline dystrophy [1652].

A search with a battery of carcinogens [350] indicated that none are substrates for bioactivation (Xiao, Y., and Guengerich, F.P., unpublished results).

9.7.33.5 Knowledge About Active Site

No definite information is available, although at least one homology model has been reported [1655].

9.7.33.6 Inhibitors

HET0016 (an inhibitor of ω-hydroxylation reactions of other subfamily 4A P450s) inhibited P450 4V2-catalyzed lauric acid ω-hydroxylation with an IC_{50} of 38 nM [1654].

9.7.33.7 Clinical Issues

The only clinical issue relevant to P450 4V2 is Bietti's crystalline dystrophy [1651]. This is a rare ocular disorder and a progressive disease that leads to atrophy of the retinal epithelium, constriction of the visual field, and night blindness. The role of P450 4V2 has been corroborated in a number of genetic studies [1651–1653]. At this time, the basis appears to be the accumulation of the fatty acids that are normally cleared by P450 4V2 [1652].

9.7.34 P450 4X1

9.7.34.1 Sites of Expression

P450 4X1 mRNA is found in a number of tissues, including liver, kidney, skeletal muscle, aorta, trachea, breast, ovary, and uterus [180]. Another site is brain, with P450 4X1 being found in the cerebellum, amygdala, and basal ganglia [1656]. Expression of the protein has also been detected in human liver [635].

9.7.34.2 Regulation

The only major study on regulation is work by Johnson and his associates [180] in human hepatoma HepG2 cells. The gene is regulated by the PPARα receptor, which regulates some other subfamily 4A P450s.

9.7.34.3 Genetic Variation

Apparently no work has been published on P450 4X1 polymorphism or other genetic variation.

9.7.34.4 Substrates and Reactions

The only reported substrate for P450 4X1 is anandamide (*N*-arachidonylethanolamine) [1656], with the reaction yielding the 14,15-epoxide. Arachidonic acid was also slowly converted to its 8,9- and 14,15-epoxides. A study with a battery of carcinogens [350] yielded no positive results for the activation of any carcinogen [350] by baculovirus-expressed P450 4X1 (Y. Xiao, and F. P. Guengerich, unpublished results).

9.7.34.5 Information About Active Site

Presently no information about the active site is available.

9.7.34.6 Inhibitors

No inhibitors of P450 4X1 have been reported.

9.7.34.7 Clinical Issues

At this point, no clinical issues have been identified.

9.7.35 P450 4Z1

9.7.35.1 Sites of Expression

Most of the reports of P450 4Z1 are focused on the expression of P450 4Z1 in breast cancer cells [1657, 1658]. P450 4Z1 has also been considered as a marker for prostate cancer [1659] and ovarian cancer [1660]. P450 4Z1 is also expressed in normal breast tissue [180].

9.7.35.2 Regulation

Limited information is available. Savas et al. [180] utilized T47-D and MCF-7 human mammary carcinoma cells and found considerable induction with dexamethasone or progesterone. These results implicate the glucocorticoid and progesterone receptors, and mifepristone (RU486), an inhibitor of both, blocked induction.

9.7.35.3 Polymorphism and Genetic Variation

No reports have appeared regarding polymorphism or other genetic variation at this time.

9.7.35.4 Substrate and Reactions

The only reactions reported for P450 4Z1 are ω-2, ω-3, ω-4, and ω-5 hydroxylations of lauric and myristic acids [1661]. The significance of these reactions is unclear, in that these are not very physiologically relevant in mammals, and longer-chain fatty acids were not considered.

Because of the possible relevance of P450 4Z1 to cancer, we expressed the enzyme (baculovirus system) in our own laboratory and screened a battery of carcinogens [350] for activation, but all were negative (Y. Xiao and F. P. Guengerich, unpublished results).

9.7.35.5 Structure

At this point, no information is available.

9.7.35.6 Inhibitors

No inhibition studies have been reported.

9.7.35.7 Clinical Issues

P450 4Z1 is not an issue in terms of its metabolic capability. The clinical interest in P450 4Z1 involves the use of mRNA expression as a tumor marker [180, 1226, 1657–1660].

9.7.36 P450 5A1

P450 5A1 is the classification of thromboxane synthase, which converts prostaglandin H_2 to thromboxane (Fig. 9.21). Thromboxane, the product, causes vasoconstriction and platelet aggregation, which are of considerable interest.

Search names include CYP5A1, P450 5A1, and TBXAS1 for this enzyme, with the latter dominating the literature.

9.7.36.1 Sites of Expression

P450 5A1 is expressed in platelets and also erythroleukemia cells [1663]. The enzyme is also found in human monocytes [1664], leukocytes [1665], and kidney interstitial dendritic reticulum cells surrounding the tubules [1666]. Some expression is also seen in lung and liver [1664].

9.7.36.2 Regulation

As one might expect from its physiological function, P450 5A1 is a highly regulated enzyme. Dexamethasone induces P450 5A1 in human monocytes [1664]. Phorbol esters also induce P450 5A1 (e.g., 12-*O*-tetradecanoyl-phorbol-13-acetate) in human erythroleukemia cells [1667]. Patients with systemic sclerosis showed sixfold enhanced levels of leukocyte P450 5A1 [1665].

Promoter analysis indicates a 39-bp core promoter, containing TATA and initiator elements that control transcription. Binding of the transcription factor NF-E2 is critical both for alteration of the nucleosomal structure and for activation of the P450 5A1 promoter [1668].

Further, Nrf2 has been reported to regulate P450 5A1 in human lung cells [1669]. Reduced methylation of the gene is correlated with increased expression levels (of P450 5A1) and preeclampsia [1670].

9.7.36.3 Genetic Variation

Chevalier et al. [1671] identified 11 variants in the *CYP5A1* gene, including eight missense

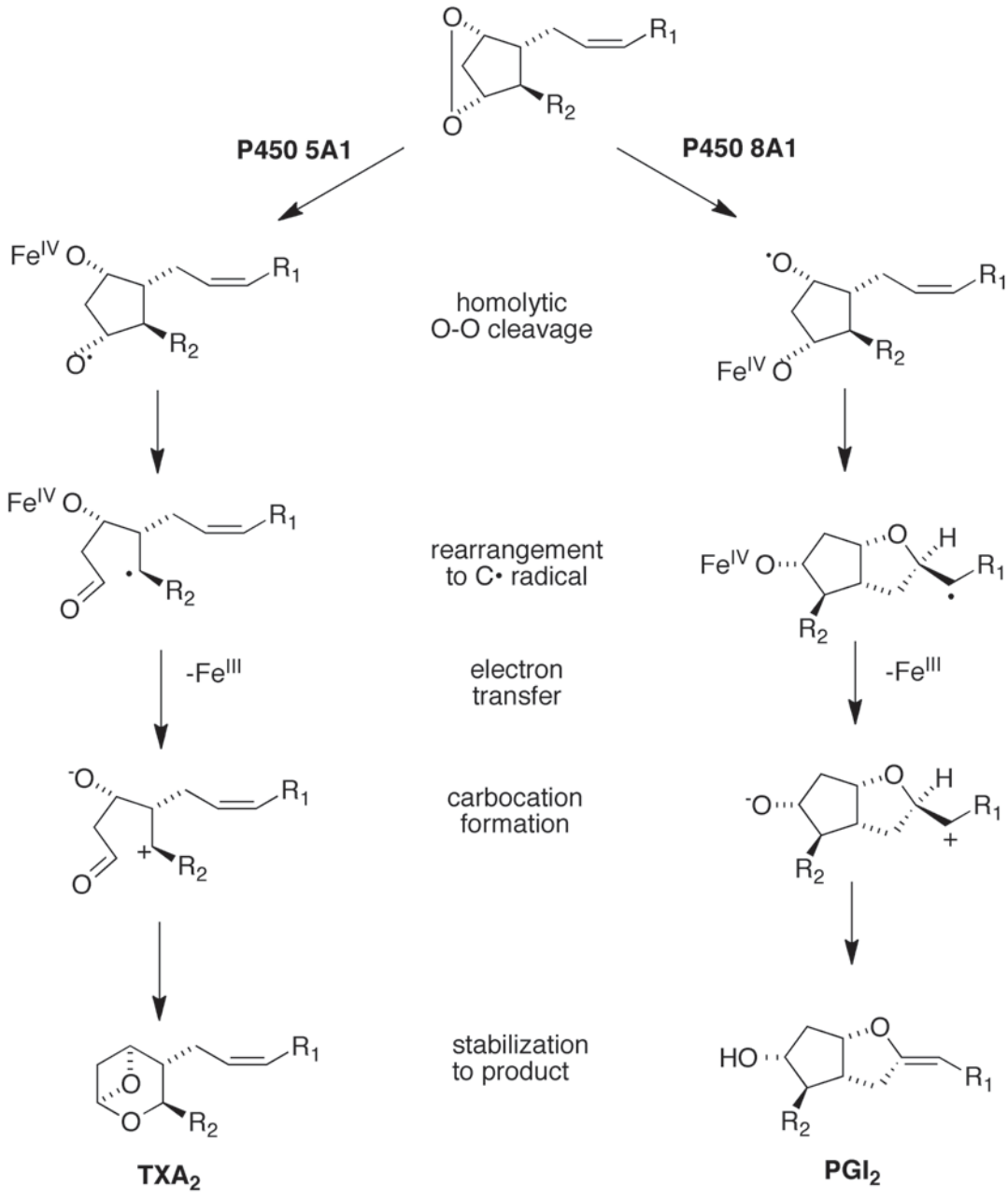

Fig. 9.21 Rearrangement of prostaglandin H_2 to prostacyclin (PGI_2) by P450 8A1 and thromboxane (TXA_2) by P450 5A1 [1662]. (With kind permission from Springer Science + Business Media: [149], Fig. 10.12)

changes in the coding region. The effects of these changes have not been reported yet. The current http://www.cypalleles.ki.se website shows 12 allelic variants reported to date, mostly those of Chevalier et al. [1672]. Racial differences have been reported between Caucasian and African American populations [1673]. Some in vitro functional characterization of variants has been reported [1674]. Polymorphisms have been associated with cerebral infection [1675].

9.7.36.4 Substrates and Reactions

The thromboxane synthase reaction has been known for many years but was shown to be a P450 by Ullrich and his associates, first in spectral studies [1676] and then by purification [1677]. With the purified enzyme or one expressed in a baculovirus system [1678], prostaglandin H_2 was converted to thromboxane A_2 and 12-hydroxyheptatrienoic acid (HHT) plus malondialdehyde, in equimolar amounts [1679] (Fig. 9.21). Prostaglandin G_2 was transformed to malondialdehyde and the corresponding 15- and 12-hydroperoxy products. Prostaglandin H_1 was enzymatically transformed into 12(*L*)-hydroxy-8,10-heptadecadienoic acid, and prostaglandin H_3 yielded thromboxane B_3 and 12(*L*)-hydroxy-5,8,10,14-heptadecatetraenoic acid [1679] (Fig. 9.21).

These are all rearrangement reactions, not involving input of O_2 or electrons from pyridine nucleotides. The reaction mechanism has been reviewed [1680]. The reaction of the "oxygen-surrogate" iodosylbenzene with a P450 5A1-containing preparation and the stable prostaglandin H_2 analog 15(*S*)-hydroxy-11α,9α-epoxymethano-5(*Z*),13(*E*)-prostadienoic acid (U46619) yielded three oxidation products (that could also be formed in a similar system using rat liver microsomes) [1681]. These and other studies led Hecker and Ullrich [1682] to propose a mechanism involving homolytic cleavage of the prostaglandin endoperoxide (with the Fe^{IV} bonded to one oxygen and the other oxygen bearing a radical), transfer of the radical to a carbon, further electron transfer to generate Fe^{III} plus a carbocation, and collapse of the *bis*-ionic structure to yield thromboxane A_2 (Fig. 9.21) [1662, 1680]. Fragmentation competes with the electron transfer step to also yield malondialdehyde and heptatrienoic acid [1662].

9.7.36.5 Structure

Although a more soluble form of P450 5A1 has been engineered [1683], no reports of crystal structures have appeared. Several spectroscopic [1684, 1685] and modeling [1686] studies have been published. One conclusion has been that the active site is relatively large and hydrophobic [1685]. As indicated, the protein does not bind NADPH-P450 reductase. Presumably the active site is rather specific, although iodosylbenzene could be utilized as an oxygen surrogate [1681].

9.7.36.6 Inhibitors

Thromboxane synthase inhibitors have been a matter of interest for many years because of their potential use in preventing plugs of platelets, and efforts at development preceded the characterization of the enzyme as a P450 [1687–1689]. Many of these inhibitors have a basic nitrogen atom that binds to the P450 5A1 heme [1690].

For a review of both P450 5A1 inhibitors and thromboxane receptors, which have been used together, see [1691]. Quantitative structure–activity relationships of both have been reviewed [1692]. Among the uses for P450 5A1 inhibitors are platelet function [1693], atherosclerosis [1694], inflammatory bowel disease [1695], lung cancer [1696], and production of hepatitis C virus (in a humanized mouse model) [1697].

9.7.36.7 Clinical Issues

As indicated earlier, platelet aggregation due to thromboxanes is important, but overproduction can yield clots, so control of homeostasis is desirable. Much of the clinical interest is in inhibiting this enzyme. Most of the issues are with cardiovascular diseases related to platelet function. Genetic variations have been considered in relation to aspirin tolerance in asthmatics [1698] and acute urticaria induced by nonsteroidal anti-inflammatory drugs [1699]. P450 5A1 signaling relationships with cancer have also been considered [1700, 1701].

9.7.37 P450 7A1

P450 7A1 catalyzes cholesterol 7α-hydroxylation, the rate-limiting step in bile acid synthesis. The enzyme was isolated from rabbit and rat liver [1702, 1703] and partially purified from human liver [1704]; the cDNA was cloned by several groups in 1990 [1705–1707].

9.7.37.1 Sites of Expression

Apparently the only major site of P450 7A1 expression is the liver. The *CYP7A1* gene is on chromosome 8q11–q12 and contains recognition

sequences for a number of liver-specific transcription factors (*vide infra*) [1708–1710].

The level of the enzyme in liver appears to be similar to some of the low-to-moderately abundant xenobiotic-metabolizing enzymes in liver.

9.7.37.2 Regulation

The regulation of the *CYP7A1* gene is very complex, as might be expected from the important physiological role this enzyme plays.

P450 7A1 activity has long been known to be upregulated by dietary cholesterol in most animal models [1706], although there are some exceptions [1711]. Feeding rats the competitive inhibitor 7-oxocholesterol led to reduced bile acid synthesis (due to inhibition) and a compensatory increase in P450 7A1 synthesis [1712]. Chiang [1713] identified a bile acid-responsive element in the *CYP7A1* promoter.

Studies with *CYP7A1*-knockout mice show that this reaction (cholesterol 7α-hydroxylation) is essential for proper absorption of dietary lipids and fat-soluble vitamins in newborn mice but not for maintenance of cholesterol and lipid levels [1714]. The mice exhibit a complex phenotype with abnormal lipid excretion, skin pathologies, and behavioral irregularities. The cholesterol levels were not altered. Interestingly, vitamin D_3 and E levels were low to undetectable.

A new era in the regulation of P450 7A1 began with reports of the involvement of some of the orphan steroid receptors. The proximal promoter region interacts with LXRα. The oxysterols 24(*S*)-hydroxycholesterol and 24(*S*)-epoxycholesterol activate LXRα (and LXRβ) [1715]. Further, mice devoid of LXRα fail to induce *CYP7A1* transcription [1716]. Two other proteins, farnesoid X receptor (FXR) and cleavage and polyadenylation factor (CPF), are also involved [1717–1719]. Chenodeoxycholate, a bile acid derived from cholesterol, interacts with FXR to suppress *CYP7A1* transcription [1720]. However, the action of FXR has been reported to be indirect [1720]. PXR binds lithocholic acid and downregulates *CYP7A1* [1721]. Thus, cholesterol metabolites control their synthesis in the liver through feedback suppression of *CYP7A1* [1717]. Hylemon [1722] concluded that the dominant factor is LXRα. CPF binds to the promoter (as a monomer) and leads to *CYP7A1* transcription [1719].

Other studies have addressed the role of PPARα in P450 7A1 downregulation [1723]. However, differences between human and mice gene responses have been observed, with the mouse gene showing an enhanced response to ligands because of an additional binding site [1724] (further, humans have much less PPARα than rodents [1725]). Chiang [1726] analyzed the PPARα response and provided evidence that the downregulation by the PPARα-agonist complex is due to competition with HNF-4 for the DR-1 sequence.

The regulation of P450 7A1 by other factors has been considered. Downregulation by TNFα has been interpreted in the context of MEKK1, an upstream nitrogen-activated protein kinase, affecting HNF-4 [1727]. The same mechanism may be involved in the repression by endotoxin and interleukin-1 [1728]. A novel *CYP7A1* site appears to be involved in the repression of *CYP7A1* by thyroid hormone (T_3) [1729]. Studies with rats indicate differences in the regulation of P450 7A1 and P450 27A1, a sterol 27-hydroxylase [1730]. Human *CYP7A1* expression is also repressed by insulin and phorbol esters [1731]. Estrogen (100 µg/kg/week) increased hepatic cholesterol 7α-hydroxylation 2.7-fold in ovariectomized baboons [1732].

In addition to the mouse *CYP7A1* knockouts, work has been done with overexpression in mice [1733, 1734]. The mice did not exhibit altered cholesterol levels [1734]. The lack of an LXR element in a region (−56 to −49) of the human promoter may dictate some of the differences seen in mouse and human models. With regard to humans, one study of biopsy samples from gallstone patients led to the conclusion that there was no correlation between levels of total bile acids and P450 7A1 activity [1735]. A correlation was seen with levels of CDCA.

A long-standing observation from rodent studies is the apparent circadian rhythm of P450 7A1 [1736]. This phenomenon has been suggested to be indicative of a short half-life of the enzyme [1737, 1738]. The phenomenon has also been reported in nonhuman primates [1739]. The circadian rhythm can be demonstrated at the level of

actual P450 7A1 in rats [1740]. The molecular mechanism of the rhythm is still not clear. One aspect is the reported instability of P450 7A1 in microsomes (in vitro), with a $t_{1/2}$ of ~1–2 h in humans and rats [1741]. Alternatively, the mRNA has a short $t_{1/2}$ and the circadian rhythm can be seen at the mRNA level [1742]. Another unresolved aspect of P450 7A1 research is the issue of phosphorylation, postulated early in the field [1743]. In vitro experiments with microsomes show some effects of various treatments [1744–1746], although the in vivo significance is yet unclear (*vide infra*).

Since the last edition of this chapter was published [149], the complexity of P450 7A1 regulation has increased. The hepatocyte growth factor signaling pathway has been shown to inhibit P450 7A1 expression [1747]. Fibrates inhibit P450 7A1 expression in culture via the LXRα and PPARα heterodimers [1748]. The LXR repression of P450 7A1 expression in human hepatocytes contrasts with the stimulation seen in rodent liver [1749]. The species selectivity of P450 7A1 gene regulation has also been noted by others [1750] (*vide supra*).

Glucose stimulates P450 7A1 gene transcription in human hepatocytes [1751]. Insulin regulates P450 7A1 expression (in human hepatocytes) via Forkhead box O1 and SREBP 1c [1752]. SREBP-1c is responsible for mediating the functional interaction of HNF-4 and PPARγ coactivator 1α [1753, 1754].

The coactivator PGC-1α also activates P450 7A1 expression [1755]. Under-expression of both PGC-1α and SRC1 impairs HNF-4α and promotes dedifferentiation in human hepatoma cells and downregulation of P450 7A1 [1756].

Retinoic acid represses P450 7A1 expression in human hepatocytes and HepG2 cells via both FXR/RXR-dependent and independent mechanisms [1757]. Glycosylation of fibroblast growth factor receptor 4 (GRF4) was shown to downregulate P450 7A1 [1758]. Ligand-dependent regulation of the orphan nuclear receptor small heterodimer partner (SHP) is involved in repression of P450 7A1 [1759]. Further, HNF-4α and liver receptor homolog-1 (LRH-1) cooperate in the regulation of P450 7A1 [1760]. Thyroid hormone was reported to regulate human P450 7A1 in humanized mice [1761].

A possible role of microRNA in P450 7A1 regulation was reported [1747].

Another aspect of P450 7A1 regulation is phosphorylation. The topic has been reviewed by Stroup [1762, 1763]. Multiple sites of phosphorylation have been proposed [1762], although a proteomic search did not reveal any phosphorylated P450 7A1 peptides [297].

9.7.37.3 Genetic Variation

Gentic variations in the coding and noncoding regions of the *CYP7A1* gene are known [1764]. Some have been associated with clinical changes [1765] but others have not [1766].

A promoter variant has been considered with plant sterols and shown to yield increased P450 7A1 transcriptional activity in (transfected) HepG2 cells [1767]. Genetic variants have also been considered in regard to colorectal [1768] and gallbladder [1769, 1770] cancers.

9.7.37.4 Substrates and Reactions

The classic reaction of P450 7A1 is cholesterol 7α-hydroxylation [37], and esterified cholesterol is not a substrate [1771]. The enzyme also catalyzes the 7α-hydroxylation of 24-hydroxycholesterol, with preference for the (*S*)-isomer [1772]. 7α-Hydroxylation (with recombinant human P450 7A1) was observed with 20(*S*)-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol [1773]. The relevance of the activity towards 25(*S*)-hydroxycholesterol is unknown compared to P450 39A1 [1774].

The P450 7A1-catalyzed 7α-hydroxylation of cholesterol appears to be among the fastest reactions for a mammalian P450, with k_{cat} ~ 190 min^{-1} and k_{cat}/K_m of ~2.4×10^6 M^{-1}/s [1775] (P450 21A2 is also a very efficient enzyme, *vide infra*). Pre-steady-state kinetic analysis and kinetic deuterium isotope effects were used to establish that the reduction of ferric iron is the rate-limiting step in the 7α-hydroxylation [1775].

In addition to cholesterol, several other sterols bind to P450 7A1 and show some conversion to (uncharacterized) oxidation products, i.e., epicholesterols, 5-androstene-3β-ol [1776].

P450 7A1 has also been demonstrated to convert lathosterol to 7-ketolathosterol (the immediate precursor of cholesterol in the normal pathway) to 7-ketocholesterol and a trace of the 7,8-epoxide [1777]. The reaction with Δ^7-dehydrocholesterol is proposed to be responsible for the high level of the oxysterol 7-ketocholesterol in individuals with Smith–Lemli–Opitz syndrome [1777], and the ketone is formed in a "direct' reaction (carbocationic intermediate, with hydride transfer) rather than via rearrangement of the epoxide [1777]. The relevance of this reaction has been demonstrated in Smith–Lemli–Optiz syndrome and cerebrotendinous xanthomatosis patients [1778].

9.7.37.5 Structure

The binding of several cholesterol analogs was used to propose a homology model [1776, 1779]. The region 214–227 has been postulated to interact with the membrane and to serve as a substrate access channel [1780]. Mutations in the regions yielded some changes in kinetic parameters towards cholesterol.

X-ray crystal structures of human P450 7A1 are available, unliganded and with cholest-4-en-3-one and 7-ketocholesterol (PDB 3DAX, 3SNS, 3V8D, http://www.rscb.org, Strushkevich et al., online but not published in periodicals).

9.7.37.6 Inhibitors

Limited information about inhibitors is available. As indicated earlier, 7-ketocholesterol is a (competitive) inhibitor [1712].

9.7.37.7 Clinical Issues

P450 7A1 has been a topic of considerable interest in the areas of hepatology and gastroenterology. Efforts to use drugs to utilize P450 7A1 to lower cholesterol have been reviewed [1781].

The hypersecretion of cholesterol in obesity does not appear to be due to reduced 7α-hydroxylation [1782]. Coffee terpenes (e.g., cafestol) inhibit P450 7A1 and also raise cholesterol levels [1783], although it is not clear that the two phenomena are linked. The complex regulation of P450 7A1 makes interpretation of experiments difficult. Overexpression of P450 7A1 in HepG2 cells increased bile acid synthesis but led to decreased HMG-CoA reductase activity (rate-limiting step in cholesterol biosynthesis) [1784].

Alterations in P450 7A1 were not seen in hypo- or hyperthyroidism [1785].

A 10-week-old child with a stop codon mutation and lacking P450 7A1 presented with severe cholestasis, cirrhosis, and liver synthetic failure [1765]. A frameshift leading to (homozygous) lack of P450 7A1 was associated with high LDL cholesterol but not total cholesterol [1786]. Heterozygotes were also hyperlipidemic. However, Beigneux et al. [1787] have discussed some of the caveats associated with interpretation of results of family and experimental studies with P450 7A1.

Several studies have been published on the effects of genetic variants on plasma lipid composition [1788–1790] and also on response to a high-fat diet [1791, 1792]. Genetic variations have also been linked to responses to fibrates [1793] and statins [1794–1796].

Genetic variations in P450 7A1 have also been related to gallstone disease [1797], bile acid synthesis rates following ileal resection [1798], risk of neuromyelitus optica [1799], and hypertension [1800].

9.7.38 P450 7B1

P450 7B1, a microsomal P450, was discovered as an "alternative" 7α-hydroxylase that used oxysterols as substrates [1801, 1802]. The enzyme is conserved in nature, even in a Japanese fire-bellied newt and the fungus *Aspergillus niger* [1802].

9.7.38.1 Sites of Expression

P450 7B1 mRNA is found not only in liver but also in the steroidogenic tissues testes, ovary, and prostate, in brain, and in colon, kidney, and small intestine [1803, 1804]. The tissue specificity of expression varies among species. Human mRNA levels are highest in kidney and brain, but expression is also seen in tissues involved in steroid biosynthesis (testes, ovary, prostate) and bile acid synthesis (liver) and reabsorption (colon, small

intestine) [1805]. As will be seen later, the clinical issues are mainly associated with the lack of the enzyme in liver and brain [1802]. P450 7B1 is overexpressed in prostate during progression of prostate adenocarcinoma [1806]. Evidence was presented for the existence of multiple sterol 7α-hydroxylases [1801, 1807], and a novel rat brain gene was identified [1808, 1809]. Although much of the literature involves animal models, a considerable amount of interest has been generated regarding human P450 7B1 because of its role in multiple diseases [1802].

9.7.38.2 Regulation

In mice, a gender variation has been reported, along with hormonal regulation, but whether any of this applies to humans is unknown. Expression is regulated by androgens and estrogens in prostate cancer LNCaP cells [1810] and HEK293 cells [1810]. A possible role for estrogenic regulation of P450 7B1 controlling DHEA levels in human tissues has been proposed [1810]. HNF-1α and Sp1 regulation has been reported [1811–1813]. In mice, the *CYB7B1* gene is regulated by RORα and LXR [1814], but this has not been confirmed in a human-based system. P450 7B1 expression was upregulated in (human) prostate during prostatic adenocarcinoma [1806]. Human *CYP7B1* gene expression is controlled by SREBP [1754].

9.7.38.3 Genetic Variation

At least 17 different variants have been found in >20 unrelated families due to the significance of diseases (*vide infra*) [1765, 1802, 1815–1818]. Not surprisingly, there are ethnic differences [1819]. A number of variants have been identified in patients with hereditary spastic paraplegia type 5 [1815, 1820–1826 and liver failure [1827]. Other variants have been identified but not necessarily related to diseases [1819, 1828].

9.7.38.4 Substrates and Reactions

Human P450 7B1 has not been purified or characterized in kinetic terms, and much of what is concluded is based on inference from animal models [1802]. The oxidations are 7α- and 6α-hydroxylation of several steroids and oxysterols (e.g., 25- and 27-hydroxycholesterol, 7α-hydroxylation of pregnenolone, DHEA, 25-hydroxycholesterol, and 27-hydroxycholesterol and 6α-hydroxylation of 5α-androstane-3β, 17β-diol [1802–1804]. Other reported substrates include testosterone and 17β-estradiol [1803, 1804]. DHEA is a "prohormone," secreted by the adrenals, and undergoes tissue-specific metabolism to yield multiple products that have a variety of biological effects [1803, 1804], producing compounds important in cognition, behavior, and immune response [1808, 1829].

5α-Androstene-3β,17β-diol ("anediol") undergoes 6α-hydroxylation, and this reaction occurs in prostate. The rest of the reactions are all 7α-hydroxylations. In the liver, the 7α-hydroxylations of 25- and 27-hydroxycholesterol are associated with bile acid synthesis. In the brain, 7α-hydroxylation of pregnenolone and DHEA is part of steroid hormone metabolism. Metabolism of ER ligands involves 7α-hydroxylation of DHEA in the prostate and 27-hydroxycholesterol in the vasculature (as well as 6α-hydroxylation of 5α-androstene-3β,17β-diol). Immunoglobulin production (in immune cells) involves 7α-hydroxylation of 25-hydroxycholesterol. Another known reaction is the 7α-hydroxylation of 5α-androstene-3β,17β-diol ("enediol"), at least with the rat enzyme.

9.7.38.5 Structure

No structures of P450 7B1 are available, in that the enzyme has not been reported to be purified yet. At least two homology models have appeared [1820, 1830].

9.7.38.6 Inhibitors

No specific inhibitors of P450 7B1 have been reported. A nonselective inhibitor, clotrimazole, was used to inhibit the rat enzyme in prostate fractions [1831]. Schwarz et al. [1809] note that nafimidone has been reported to inhibit the mouse enzyme but not the human.

9.7.38.7 Clinical Issues

Stiles et al. [1802] reviewed the two major issues, both of which are related to genetic variations. One is liver failure in children and the other is

neuropathy in adults. These seemingly unrelated diseases may be understood in the variety of P450 7B1 substrates and the diversity of biological actions of steroids. The biological roles of P450 7B1 include hepatic bile salt synthesis (25- and 27-hydroxycholesterol being substrates), brain steroid hormone metabolism (pregnenolone and DHEA being substrates), prostate and vasculature metabolism of ER ligands (5α-androstane-3β,17β-diol, DHEA, and 27-hydroxycholesterol being substrates), and immunoglobulin production in immune cells (25-hydroxycholesterol being substrate). Overall, there are two driving issues, the production of appropriate steroid hormones and the removal of deleterious oxysterols, depending upon the site.

The two major clinical issues are liver failure in children (due to genetic insufficiency) [1802, 1827, 1832, 1833], and neuropathy (in adults), particularly the autosomal recessive disorder spastic paraplegia type 5 [1802, 1815–1818, 1824, 1825]. Possible association with Alzheimer's disease has also been reported [1834]. An association with rheumatoid arthritis has been considered [1835]. P450 7B1 has also been mentioned regarding (low activity) and the promotion of cell-autonomous ER-positive breast cancer [1836].

9.7.39 P450 8A1

Prostacyclin (prostaglandin I_2) has strong vasodilation and anti-aggregation effects on platelets, and the imbalance of prostacyclin and thromboxane A_2 (product of P450 5A1) is a factor in several diseases, e.g., myocardial infarction, stroke, atherosclerosis [1837, 1838]. The reaction yielding prostacyclin from prostaglandin H_2 is another "internal" oxygen transfer, without the input of O_2 and electrons from NADPH (Fig. 9.21), and the involvement of a P450 was not immediately obvious. Ullrich hypothesized P450 involvement on the basis of spectral interaction studies [1839]. DeWitt and Smith [1840] used a monoclonal antibody to purify catalytically active prostacyclin synthase from bovine aorta and demonstrated a P450 $Fe^{2+}\cdot CO$ spectrum. Subsequently P450 8A1 was cloned from bovine endothelial cells [1841].

9.7.39.1 Sites of Expression

A human P450 8A1 cDNA was cloned from aorta endothelial cells by the Tanabe laboratory [1838]. The mRNA is widely expressed in human tissues, including ovary, heart, skeletal muscle, lung, prostate [1838], and umbilical vein [1842]. There is also localization in the brain, including neurons [1843, 1844]. Another site of expression is fallopian tubes, with expression in luminal epithelia, tubal smooth muscle, vascular endothelial cells, and vascular smooth muscle cells [1845].

9.7.39.2 Regulation

P450 8A1 is constitutively expressed in human endothelial cells [1842]. The human *CYP8A1* gene (chromosome 20) has ten exons [1846–1848] and has consensus sequences for Sp1, activating protein-2 (AP-2), an interferon-γ response element, GATA NFκB, a CACCC box, glucocorticoid receptor, and a shear stress-responsive element (GAGACC) [1846]. Whether or not all of these are functional and how they interact to maintain constitutive expression is not well understood yet.

Hypermethylation of the promoter has been reported as a frequent event in colorectal cancer [1849].

One posttranslational aspect of regulation is redox control of P450 8A1. Peroxynitrite causes nitration of Tyr-430 [1850], causing inactivation due to steric hindrance of the active site [1851]. This nitration has been reported to be associated with enhanced retinal cell apoptosis in diabetes [1852].

9.7.39.3 Genetic Variation

Variants have been of interest because of disease relevance. At least 14 alleles have been reported, yielding four different proteins (http://www.cypalleles.ki.se). Haplotypes have been considered in the context of essential and thromboembolic preliminary hypertension [1853, 1854], myocardial infarction [1855], left main coronary artery disease [1856], and cardiovascular disease in general [1857].

In the 5′-region, these are variants involving a variable number of tandem repeats (VNTR) that affect transcription, as demonstrated in reporter systems in vitro [1672]. An association between this VNTR polymorphism and cerebral infarction has been reported [1858].

An SNV in exon 8 has been reported to be linked to myocardial infarction, although no amino acid change occurs [1859]. However, the VNTR variation does not appear to be related to essential hypertension [1860], nor does the 5′-flanking region SNV T192G [1861]. However, a novel splicing variation leading to skipping of exon 9 has been linked to hypertension [1862].

9.7.39.4 Substrates and Reactions

P450 8A1 has a very limited catalytic specificity, functioning only as the prostacyclin synthase (Fig. 9.21). Prostaglandins G_2, H_2, 13(*S*)-hydroxy H_2, 15-keto H_2, and H_3 are isomerized to the corresponding prostacyclins [1682]. Spectral binding studies with 9,11-epoxymethano prostaglandins F_2 and $F_2\alpha$ lead to the view that the binding juxtaposition is the key determinant in distinguishing the courses of catalysis by P450s 5A1 and 8A1 [1682]. A mechanism consistent with available data has been proposed (Fig. 9.23) [1662, 1682].

Yeh et al. [1680] used 15-hydroperoxyeicosatetraenoic acid (15-HPETE) as a substrate for P450 8A1 and found both hemolytic (15-ketoeicosatetraenoic acid) and heterolytic (15-hydroxyeicosatetraenoic acid) products, with the former reaction accounting for ~80 % of the total.

9.7.39.5 Structure

A crystal structure of human P450 8A1 was reported by Chiang et al. [1863] in 2006. This structure did not include a substrate. In 2008, another structure was published by the same group, with a substrate (U51605) analog and an inhibitor (minoxidil) [1864]. Relative to the unliganded molecule, conformational changes were observed at the proximal side of and in the heme itself.

Other work has been on membrane topology, and antibody studies indicate that P450 8A1 is mainly exposed on the cytoplasmic site of the endoplasmic reticulum with a single transmembrane anchor [1865, 1866]. The (unstable) substrate, prostaglandin H_2, is produced in the lumen and apparently passes through the membrane to reach P450 8A1.

9.7.39.6 Inhibitors

Relatively little interest has been shown in development of drugs that inhibit P450 8A1 because inhibition is generally considered to be deleterious. Phenylbutazone has been reported to inhibit [1867].

The prostaglandin synthase inhibitor rofecoxib (Vioxx®, now withdrawn from the market) was reported to inhibit P450 8A1 [1868].

P450 8A1 is slowly inactivated during the normal reaction itself, apparently by one of the reactive intermediates in the catalytic cycle (Fig. 9.23) [1869]. A $k_{inactivation}$ of 0.06 s^{-1} was reported [1869].

Peroxynitrite is a powerful inhibitor of P450 8A1, with a reported K_I of 50 nM [1870]. Peroxynitrite is formed by the chemical reaction of NO·and O_2^- [1871]. The mechanism is believed to involve tyrosine nitration [1872], and recently Tyr430 has been implicated as the site of nitration [1873].

9.7.39.7 Clinical Issues

As mentioned earlier, prostacyclin is a powerful vasodilator and inhibits platelet adhesion and undesired cell growth. Although this view may be overly simplistic, prostacyclins are a counterbalance to thromboxanes in a “yin and yang” relationship. Thus, the action of P450 8A1 balances that of P450 5A1. Several of the genetic variants (Sect. 7.39.3, *vide supra*) have been related to diseases, particularly cardiovascular disease [1874].

Decreased expression of P450 8A1 has been reported in severe pulmonary hypertension [1875]. With regard to general cardiovascular disease, a study of Japanese subjects associated the VNTR variation with hypertension (odds ratio 1.9) [1876]. Individuals with three to four repeats had less promoter activity and higher risk. In experimental studies, the overexpression of P450 8A1 in transgenic mice protected against the development of hypoxic pulmonary hyper-

tension [1877]. In another study, the expression of human P450 8A1 in the carotid arteries of rats after arterial balloon injury (using a virus) led to increased synthesis of prostacyclin and to reduced neointimal formation [1878].

P450 8A1 also has relevance in cancer treatment. Transfection of colon adenocarcinoma cells with P450 8A1 led to slower growth and reduced vascular development following inoculation into syngeneic mice [1879]. P450 8A1 has also been considered in the context of cancer as a target in non-small cell lung cancer [1700].

Finally, antibodies in the sera of some patients with hypersensitivity reactions to phenytoin and carbamazepine recognize rat P450 3A1 but not human P450 3A [1880]. The antisera also recognizes P450s 8A1 and 51A1, although relationships of etiology and causality are unclear.

9.7.40 P450 8B1

9.7.40.1 Sites of Expression

P450 8B1 is a sterol 12α-hydroxylase expressed in the liver. The human *CYP8B1* gene was characterized on the basis of the rabbit and mouse orthologs [1881]. Of interest is the finding that this gene is devoid of introns, unique for this gene among the P450 family [1881].

9.7.40.2 Regulation

Regulation of the gene is of interest, in that P450 8B1 catalyzes the synthesis of cholic acid and controls the ratio of cholic acid to CDCA in the bile [1882]. Much of what has been reported in the literature is with animal models. HNF4α activates human *CYP8B1* expression in HepG2 cells [1882]. Bile acids and FXR downregulate HNFα expression. Inflammation in liver cells causes increased synthesis of α_1-antitrypsin, a serum protease inhibitor, and in a derived peptide (C-36). C-36 appears to interact with the α_1-fetoprotein transcription factor (FTF) site in the human *CYP8B1* promoter, inducing a conformational change to lower DNA binding ability, and suppressing the transcription of the *CYP8B1* (and *CYP7A1*) genes [1883, 1884]. HNFα could overcome the inhibitory effects of FTF and bile acids [1884]. Thus, regulation of P450 8B1 is involved in bile acid feedback inhibition.

Ligand-dependent regulation of the orphan nuclear receptor SHP has been reported to downregulate P450 8B1 expression is HepG2 cells [1759]. Phenobarbital regulated P450 8B1 in HepaRG cells [1885]. The corepressor GOS2 has also been reported to regulate P450 8B1 [1886]. Soy isoflavones upregulated human P450 8B1 [1887]. Based on animal models, cytokines and liver factor HNF-1α regulate P450 8B1 [1888, 1889].

The in vivo phosphorylation of P450 8B1 has been reported [297].

P450 8B1 has been reported to show circadian rhythm [1803].

9.7.40.3 Genetic Variation

Limited reports on genetic variation have appeared [1890, 1891].

9.7.40.4 Substrates and Reactions

P450 8B1 catalyzes the 12α-hydroxylation of several oxysterols, including 4β- and 7α-hydroxycholesterol and 7α, 24- and 7α,27-dihydroxycholesterol, yielding (following P450 27A1 action) the primary bile acid cholic acid [1803]. P450 8B1 controls the balance between cholic acid and CDCA, adjusting the hydrophobicity of the bile (cholic acid is more hydrophilic than CDCA). However, variations in the cholic acid to CDCA ratio do not seem to be controlled by genetic variation in P450 8B1 [1803].

9.7.40.5 Structure

No structures have been reported, and a literature search did not reveal any homology models.

9.7.40.6 Inhibitors

No selective inhibitors have been published. CDCA has been reported to inhibit P450 8B1. A limitation of inhibition of P450 8B1 activity is that a decrease in the cholic acid to CDCA ratio might cause hepatotoxicity, which was observed in patients treated with CDCA for gallstones [1803, 1892].

9.7.40.7 Clinical Issues

An SNV in the CYP8B1 gene has been associated with gallstone disease in a Han Chinese population [1890]. However, P450 8B1 has been reported to have a smaller effect on bile acid synthesis than P450 7A1 in human liver [1893].

9.7.41 P450 11A1

P450 11A1 is the enzyme involved in the initiation of hormonal steroid synthesis (Fig. 9.12). It catalyzes the conversion of cholesterol to pregnenolone by side-chain cleavage and has been referred to in the older literature as $P450_{scc}$ or cholesterol desmolase. The enzyme was first purified from bovine adrenal cortex mitochondria [1894]. The human gene was cloned by Omura and Fujii-Kuriyama in 1987 [1895] and includes nine exons. Of historical significance is the fact that this P450 only contains a single cysteine and further establishes the position of the heme thiolate peptide in P450s, extending the work on the location from the original crystal structure of bacterial P450 101A1 [1896].

9.7.41.1 Sites of Expression

P450 11A1 is found primarily in steroidogenic tissues, including adrenal cortex and gonads, including ovary (corpus luteum [1897, 1898] and theca interna cells [1899] and others [1900]). Of interest are reports of P450 11A1 in brain [1901–1904] and pancreas [1905].

P450 11A1 is one of several P450s localized in the mitochondria (Table 9.2, Fig. 9.12). Studies with the bovine enzyme demonstrated that P450 11A1, synthesized on ribosomes in the cytosol, is imported into mitochondria without processing of the amino terminal extension peptide [1906]. The protein moves to the mitochondrial inner membrane and is then cleaved to yield the mature form [1906]. Alteration of the basic amino acid residues of the N terminus resulted in less efficient mitochondrial import [1907]. Miller and his associates constructed vectors that could be used to direct P450 11A1 to the endoplasmic reticulum and found that the enzyme was inactive [1908]. The membrane environment was concluded to be more important in modulating catalytic function than the nature of the electron transfer partners.

9.7.41.2 Regulation

The regulation of P450 11A1 is relatively complex, as might be expected for the initial step in steroid formation [1900]. Moreover, the system must be able to respond to signals in many different tissues. Much of our understanding of the regulation of P450 11A1 expression is based on studies with *CYP11A1* genes of experimental animals and reinvestigated with human *CYP11A1*.

P450 11A1 has long been known to be regulated by ACTH and cyclic AMP. In the bovine *CYP11A1* gene, two Sp1-binding sites mediate cyclic AMP transcription through the protein kinase A signaling pathway, utilizing the rather ubiquitous transcription factor Sp1 [1909]. Steroidogenic factor-1 (SF-1) activates *CYP11A1* transcription through interaction with protein factors upstream [1900]. An upstream cAMP response element-binding protein (CREB)-binding region and an AP-1 site are also involved in the cyclic AMP response. Sp3 can also be involved [1910]. The TATA box drives cell type-specific cyclic AMP-dependent transcription [1911]. SF-1 also interacts with Sp1 [1912–1914]. Thus, the regulation of the human *CYP11A1* gene involves all the above factors plus an AdE element [1900]. Expression of the human gene has been shown to involve the zinc finger protein TreP-132, interacting with both CBP/p300 [1915] and SF-1 [1916]. Also, salt-inducible kinase (SIK) represses cyclic AMP-dependent protein kinase-mediated activation through the CREB basic leucine zipper domain [1917]. In human placenta, AP-2 assumes the role of SF-1 by binding to an overlapping promoter element [1918].

An analysis of the P450 11A1 promoter has been reported [1919].

The orphan nuclear receptor LRF-1 regulates P450 11A1 expression in human granulosa cells [1920]. The human transcription factor LBP-32 (also termed mammalian grainyhead, MGR, or LBP-32/MGR) has been reported to be a repressor of P450 11A1 [1921]. Cyclic AMP has been reported to stimulate SF-1-dependent expression of P4540 11A1 through homeodomain-interact-

ing protein kinase 3-mediated JNK and c-Jun phosphorylation [1922].

Abnormal expression of uncoupling protein-2 has been correlated with altered P450 11A1 expression in polycystic ovary syndrome (PCOS), the main cause of infertility in women [1923]. Further, studies in PCOS theca cells showed that basal and forskolin-stimulated P450 11A1 mRNA levels and promoter activity were increased [1924]. The transcription factor nuclear factor 1C2 regulated the basal activity of the minimal P450 11A1 promoter element. The P450 11A1 mRNA $t_{1/2}$ increased >twofold in the PCOS cells compared to normal ones. The 5′-untranslated region of the P450 11A1 mRNA conferred the added stability [1924].

9.7.41.3 Genetic Variation

Variations in *CYP11A1* can cause congenital adrenal insufficiency. Arg-353 was found to be critical in a study with an afflicted patient [1925].

The relationship of PCOS to the P450 11A1 promoter variants was already mentioned in Sect. 7.41.3. (*vide supra*). This issue has been considered in a large genetic study [1926]. Other genetic studies have been reported on P450 11A1 and PCOS [1927, 1928], including microsatellite variants [497].

Disruption of the P450 11A1 gene has been associated with premature birth, sex reversal, and adrenal failure [1929]. Genetic variations have also been linked to adrenal and gonadal deficiency [1930, 1931].

P450 11A1 variants have also been related to breast [1932, 1933] and endometrial [1934] cancers.

9.7.41.4 Substrates and Reactions

The P450 11A1 reaction proceeds in a three-step sequence, with generation of (22*R*)-20α, 22-dihydroxycholesterol as an intermediate (Fig. 9.22) [1935]. Oxidative cleavage of the diol to pregnenolone and 4-methylpentanal (isocaproic aldehyde) completes the overall reaction. The mechanism of the last step is not completely clear, but some proposals have been presented [1936–1938].

The rate of electron transfer from adrenodoxin is important and appears to be the rate-limiting step for the enzyme in human placenta [1939]. The redox potential of adrenodoxin can be varied by site-directed mutagenesis but had little effect on rates of electron transfer, consistent with the view that other factors such as protein–protein interactions are more important than the intrinsic thermodynamics [1940]. When P450s 11A1 and 11B1 are expressed together in cells, they can compete for reducing equivalents from adrenodoxin [1941]; exactly how important the competition is in tissues is unclear. Another report indicates interaction of P450 11A1 with and enhancement by cytochrome b_5 [1942], although the relevance is unclear because of the compartmental separation of P450 11A1 (mitochondria) and cytochrome b_5 (endoplasmic reticulum).

P450 11A1 has now been found to be less specific than originally thought. Vitamin D_3 is oxidized to a number of different products, on the "side chain," by P450 11A1, mainly 20-hydroxy- and 20,23-dihydroxyvitamin D_3 [1943, 1944]. In addition, 23-hydroxy-, 17α-hydroxy-, 17α,20-dihydroxy- [1944], and 20,22-dihydroxyvitamin D_3 [1945] are produced [1946]. 1α-Hydroxyvitamin D_3 can yield 1α,20-dihydroxyvitamin D_3 [1947]. Several of these products have biological activities [1945, 1948] and are formed in vivo (animal models) [1949].

7-Dehydrocholesterol is also a substrate for P450 11A1 [1943], forming five 5,7-dienal products, with mono- and dihydroxy substitution [1949]. These include the 22-hydroxyl and 20,22-dihydroxy 7-dehydrocholesterol products.

Human P450 11A1 also oxidizes ergosterol (the vitamin D_2 precursor) to two major and four minor products [1950]. The major products have been characterized as 20-hydroxy-22,23-epoxy- and 22-keto-23-hydroxyergosterol.

Finally, rat and human P450 11A1 have been implicated in the metabolism and bioactivation of a drug candidate, BMS-A ((*N*-(4-((1*H*-pyrrolo[2,3[*b*]pyridine-4-yl)oxy)-3-flurophenyl)2-oxo-1,2-dihydropyridine-3-carboxamide) [1951]. The bioactivation was implicated in the vacuolar degeneration and necrosis of the adrenal cortex of rats.

In conclusion, the specificity of P450 11A1 is not so stringent as originally thought [149]. Thus, in considering P450 11A1 in a classification such as that in Table 9.1, it joins other steroid metabo-

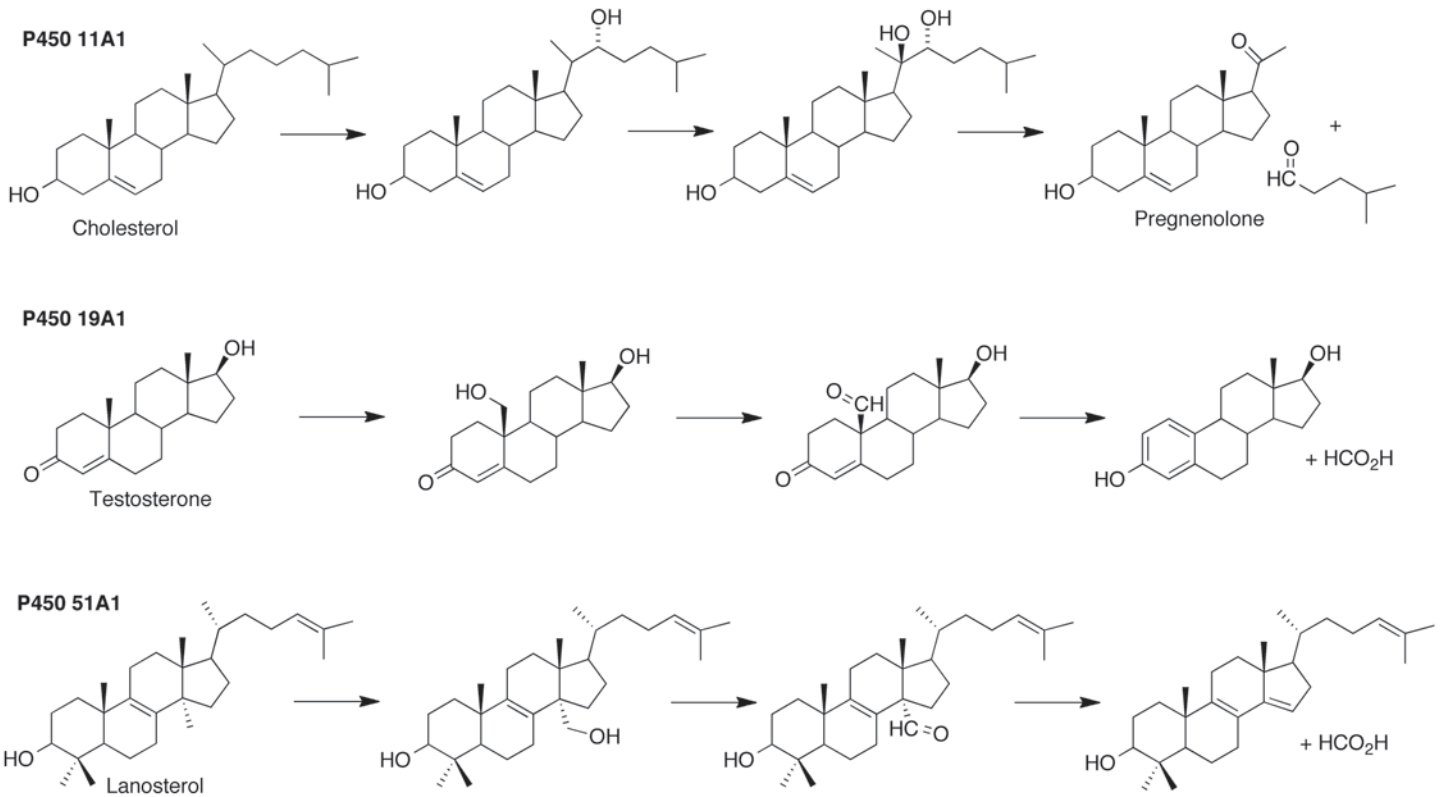

Fig. 9.22 Three multistep C–C steroid bond cleavage P450 reactions (P450s 11A1, 19A1, 51A1). Aromatization reactions catalyzed by P450 19A1: The three distinct steps are shown with the substrate testosterone. Other physiologically relevant substrates are androstenedione and 16α-hydroxytestosterone

lism P450s such as P450s 1B1, 3A4, 24A1, and 46A1 in bridging among steroid, vitamin, and xenobiotic substrates.

9.7.41.5 Structure

In 2011, Pikuleva's group [1952] reported a structure of bovine P450 11A1 bound to 22-hydroxycholesterol, the first reaction product (from cholesterol). The active site cavity can be described as a long curved tube that extends from the surface to the heme group. (A linker was used to tether adrenodoxin to P450 11A1.) The [2Fe–2S] iron cluster of adrenodoxin was positioned 17 Å away from the heme iron of P450 11A1.

A crystal structure of a human P450 11A1–adrenodoxin complex was also reported in the same year [1953], in the presence of 22-hydroxycholesterol. A structure with 20,22-dihyroxycholesterol has also been published [1953].

Limited proteolysis experiments done with P450 11A1 in *E. coli* membranes identified peptides from the putative F–G loop (residues 218–225) and the C-terminal portion of the G-helix (residues 238–250) as being involved in membrane binding [1954] (these assignments are consistent with the crystal structures).

Studies with bovine P450 11A1 indicated the significance of Lys-377 and Lys-381 in adrenodoxin binding [1955]. As indicated earlier, a mutation at Arg-353 was found to attenuate the function of P450 11A1 in a patient [1925]. Site-directed mutagenesis of human P450 11A1 (in *E. coli*) indicated that Ile-462 had some effect on kinetic parameters [1956].

9.7.41.6 Inhibitors

A number of inhibitors of P450 11A1 have been reported, although some were studied only with the bovine enzyme [1957, 1958], including some acetylenic mechanism-based inactivators [1959]. With regard to the human enzyme, there is some potential for the use of inhibitors in treatment of prostatic cancer, and prodrug forms of aminoglutethimide have been examined [1960]. Anticonvulsants have been reported to inhibit P450 11A1, but the interaction is not strong [1961].

Pikuleva's group has published a study of the inhibition of P450 11A1 by a selected set of drugs [1962]. When tested at a concentration of 10 μM (cf. 1 μM cholesterol as substrate), only ketoconazole, carbenoxolone, and selegiline inhibited >50%. No IC_{50} values were calculated, but spectral analysis yielded K_d values of 1.5 and 1.0 μM for ketoconazole and posaconazole.

9.7.41.7 Clinical Issues

Several issues are of interest. P450 11A1 insufficiency and relationship to diseases in general have been reviewed by Miller and Auchus [1963]. Because of the nature of P450 11A1 in initiating steroidogenesis, genetic variation in P450 11A1 is related to adrenal insufficiency and to congenital adrenal hyperplasia [1931, 1964–1966]. Rabbit and mouse models show the effects [1967, 1968]. *CYP11A1*-null mice die shortly after birth but can be rescued by steroid injection [1968]. ACTH levels become very high due to lack of feedback regulation by glucocorticoids. Male null mice are feminized with female external genitalia and underdeveloped male accessory sex organs. These manifestations resemble various human steroid deficiency syndromes.

Another issue is autoantibodies to P450 11A1 (and also P450 17A1) in patients with autoimmune polyglandular syndrome types I and II and Addison's disease [1969–1971]. As with other P450s recognized by autoantibodies, causal relationships between immunity and disease are unclear.

The relationship of P450 11A1 genetic variation and PCOS has already been mentioned in Sect. 7.41.3 (*vide supra*), including premature birth, sex reversal, and severe adrenal failure [1926, 1929, 1972].

Variants have also been linked to reduced P450 11A1 ovarian transcription during experimental nephrotic syndrome [1973]. Finally, P450 11A1 variants have been associated with breast [1974] and prostate [1975] cancers.

9.7.42 P450 11B1

P450s 11B1 and 11B2 differ in only 32 residues. P450 11B1 catalyzes the 11β-hydroxylation of deoxycortisol to yield cortisol (Fig. 9.23), the main glucocorticoid in the body. Deficiencies in

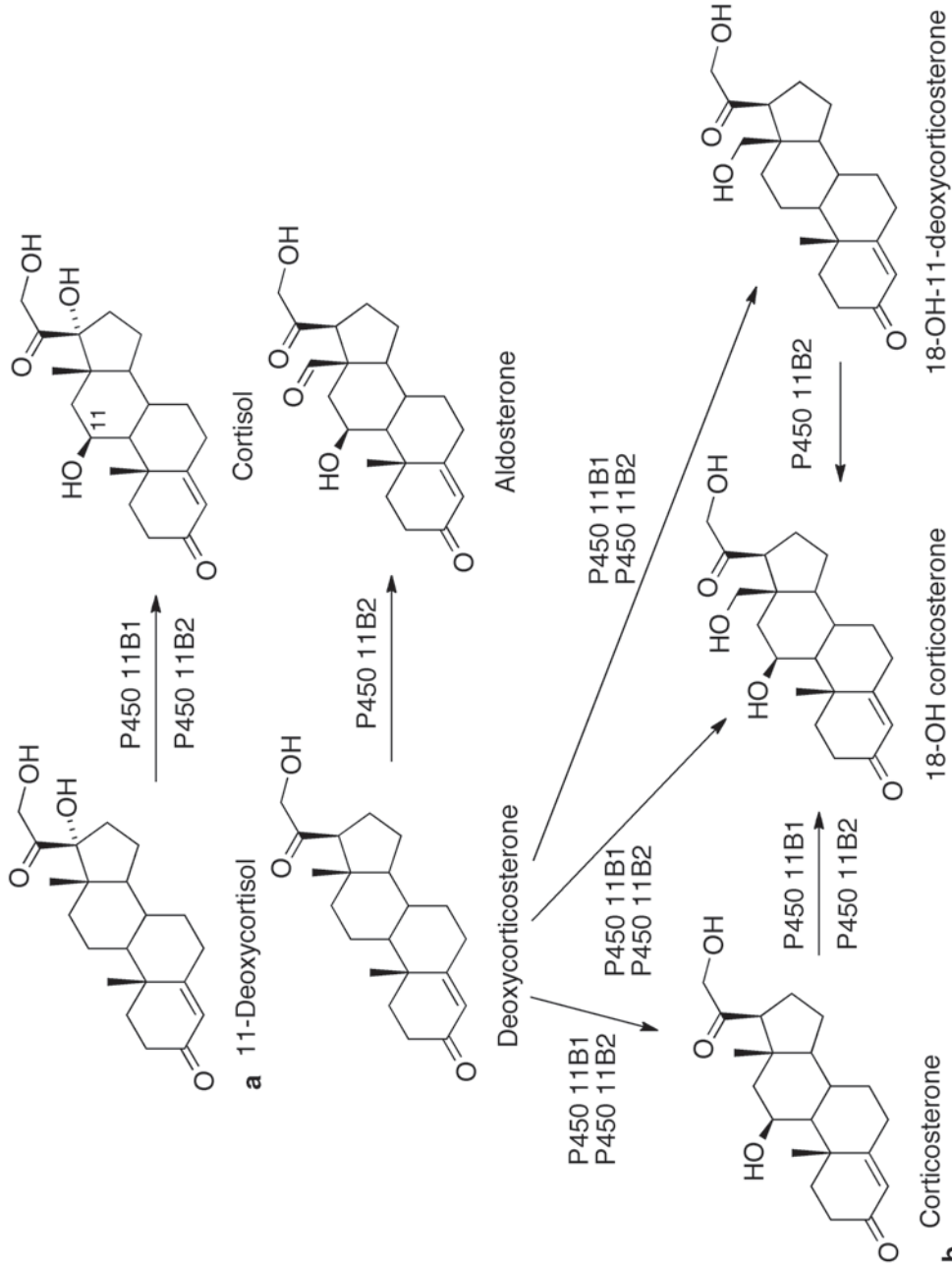

Fig. 9.23 Reactions catalyzed by P450s 11B1 and 11B2

the enzyme are known, causing congenital adrenal hyperplasia [47, 1976].

9.7.42.1 Sites of Expression

P450 11B1 is expressed in the adrenal cortex, specifically the zona fasciculata/reticularis [1976]. In rats, some expression has been detected in brain, but the relevance is not clear.

P450 11B1 is synthesized in the cytosol and directed to the mitochondria with a 24-residue N-terminal-targeting sequence (where this is lost after entry). As with the other six (exclusively) mitochondrial P450s (Table 9.2), P450 11B1 receives electrons from adrenodoxin instead of NADPH-P450 reductase.

The characterization of the *CYP11B1* gene has developed considerably in recent years. Much of the early research in this field was done with bovine adrenal glands because of the need for large amounts of material, but the bovine P450 11B1 protein has the function that P450 11B1 (11-hydroxylation) and P450 11B2 (11-hydroxylation, 18-hydroxylation, and oxidation of the 18-alcohol to an aldehyde) have in most other species, including humans [1977]. The two human genes (*CYP11B1, CYP11B2*) were characterized and clearly shown to both be essential [1978–1981].

P450 11B1 expression has also been deleted in human fetal adrenal gland, particularly in the "fetal zone" (as opposed to neocortex) [1982].

9.7.42.2 Regulation

Much of the background on regulation of P450 11B1 comes from studies with the bovine gene, which responds to ACTH and has six *cis*-acting regulatory elements [1983]. The protein (Ad4BP) that binds to one of these (Ad4) is a member of the steroid hormone receptor superfamily [1984]. Other studies by Omura [1985] indicated the cooperative nature of these elements in transcription. Work with the rat *CYP11B1* gene showed that ACTH stimulates transcription by changing composition in AP-1 factors (Fos, Jun) [1986].

The human gene also has a cyclic AMP response element (CRE) [1987]. The Ad1 element binds CRE-binding protein, activating transcription factor-1 (ATF-1), and ATF-2. SF-1 interacted at the Ad4 site (−242/−234) and is required for transcription [1987, 1988], which contrasts with the lack of response of *CYP11B2*.

ACTH modulation of transcription factors involved in regulation has been reviewed by Sewer and Waterman [181].

The orphan nuclear receptors NURR1 and NGF1B regulate P450 11B1 transcription in human H295R adrenocortical cells, and transfection with SF-1 activated P450 11B1 expression [1989].

P450 11B1 expression (mRNA and protein) was significantly higher in patients with subclinical Cushing's syndrome [1990], although the molecular basis is not known.

MicroRNA-24 was reported to regulate P450 11B1 expression in a human adrenocortical cell line [1991]. The human P450 11B1 promoter contains two Alu elements embedded in a truncated L1 element, breaking L1 into three individual fragments [1139]. The effect of Alu is blocked by a second L1 element (CYP11B1-L1.2) inserted between the first one and the conserved proximal upstream region. The CYP11B1-L1.2 element can be transcribed from the core promoter in an opposite direction (and a smaller magnitude) compared to P450 11B1. Deletion of CYP11B1-L1.2 greatly increased P450 11B1 promoter activity and restored the effect of Alu [1139]. The Ad5 and SF-1 binding elements in the proximal core promoter play a role in basal expression.

A polychlorinated biphenyl (PCB126) has been reported to upregulate P450 11B1 transcription in human adrenocortical cells due to enhancement of mRNA stability but not an AhR mechanism [1992]. The practical significance of the results is unclear, in that only concentrations ≥10 μM were used.

9.7.42.3 Genetic Variation

Many variants are known because of the relationship of the gene with congenital adrenal hyperplasia [1976]. Genetic variants related to phenotype and to inborn errors of metabolism have been reviewed [1993, 1994].

A large number of genetic variants in P450 11B1 have now been identified and related to high 18-hydroxycortisol [1995], to low 11β-hydroxylation [1996–2002], congenital adre-

nal hyperplasia [2003–2010], and hypertension [2011]. The variants include a five-base duplication [2012] and clusters of mutations in exons 6–8 [2013]. The high similarity and proximity of the *CYP11B1* and *CYP11B2* genes appear to lead to variants generated by unequal crossover and inactive chimeric products [2014–2017]. Splice donor site variants are also known [2018].

9.7.42.4 Substrates and Reactions

As indicated previously, the only reported substrate for P450 11B1 is deoxycortisol, which undergoes 11β-hydroxylation to yield cortisol (Fig. 9.12).

9.7.42.5 Structure

One of the concerns about studies on the function of particular residues in site-directed mutagenesis is that expression in some cellular systems leads to competition between P450s 11A1 and 11B1 for (adrenodoxin) reducing equivalents in cellular systems [1941]. Another issue is that human P450s 11B1 and 11B2 have been difficult to express in bacteria, so that most experiments have relied on mammalian cells (*Schizosaccharomyces pombe* has provided some success) [1976]). Information about function has also been obtained from patients' samples [1976].

Although no crystal structures of P450 11B1 have been published, structures of the highly similar P450 11B2 (one with substrate, one with an inhibitor) have appeared [2019] and, at the very least, should facilitate future modeling.

The close similarity of P450s 11B1 and 11B2 (and their reactions) has also facilitated studies. Making the changes S288G and V320A yielded an enzyme with both P450 11B1 and 11B2 activities [2020]. Changes at positions 147 [2021, 2022] and 301/355 [2023] have also had the same effect. Homology models of P450 11B1 have also been published [1976, 2008, 2024–2026], although the effects of all of the mutants known to alter function have not been systematically rationalized.

9.7.42.6 Inhibitors

Compared with some of the other steroidogenic P450s, there is some reason to develop P450 11B1 inhibitors. High levels of cortisol are associated with Cushing's syndrome [1976]. Cellular expression systems have been set up to assay for inhibitors, using measurements of concentrations of steroids [2027, 2028].

18-Vinylprogesterone and 18-ethinylprogesterone have been reported to be mechanism-based inactivators of bovine P450 11B but apparently have not been tested with human P450 11B1 [2029].

Since the previous edition of this book [149], work on more P450 11B1 inhibitors has been published [147, 2030–2036]. Some of these have been developed with the specific goals of treating prostate cancer [2037] and cardiovascular disease [2038, 2039]. One case report involves a beneficial effect in the management of an elderly patient with an androgen-producing inoperable adrenal tumor [2040].

9.7.42.7 Clinical Issues

As indicated previously, the main issue with P450 11B1 is the impaired synthesis of cortisol and congenital adrenal hyperplasia, characterized by hypertension and signs of androgen excess [2041, 2042]. The role of P450 11B1 insufficiency in congenital adrenal hyperplasia has been reviewed [2043, 2044]. The same condition is seen in a knockout mouse model [2045]. Overproduction of glucocorticoids, which could have any of several causes, including overactive P450 11B1, is associated with Cushing's syndrome [1976].

A number of genetic variations associated with disease are cited in Sect. 7.42.3 (*vide supra*). A chromosome inversion was also seen in a family [2046]. Testicular tumors in patients with P450 11B1-related congenital adrenal hyperplasia showed functional features of adrenocortical tissues [2047]. Hyperplasia of adrenal rest tissue was implicated in causing a retroperitoneal mass in a child with P450 11B1 deficiency [2048].

P450 11B1 deficiency has also been associated with hypertension [2049–2051] rhabdomyolysis [2052], virilization [2053], and prepubertal gynecomastia [2054].

9.7.43 P450 11B2

P450 11B2 is highly related to P450 11B1 (*vide supra*) and has a somewhat similar function. P450

11B2 catalyzes the 11β-hydroxylation of 11-deoxycorticosterone followed by 18-hydroxylation and 2-electron oxidation of the 18-alcohol to an aldehyde (Figs. 9.12 and 9.23). Changes in the gene can lead to corticosterone methyloxidase deficiency and hyperaldosteronism [47, 1980, 2055, 2056]. In the older literature, this P450 is sometimes termed "$P450_{aldo}$."

9.7.43.1 Sites of Expression

P450 11B2 is expressed in the adrenal cortex (zona glomerulosa) and involved in the synthesis of aldosterone (the 11β-hydroxy, 19-aldehyde product). It is a mitochondrial P450, as are the other family 11 P450s. The cDNA was first cloned from the adrenal tumor of a patient suffering from primary aldosteronism [2057]. Another early study showed higher levels of P450 11B2 in aldosterone-secreting tumors [2058].

There is some evidence for the synthesis of aldosterone outside of the adrenals, and Li et al. [2059] reported expression of P450 11B2 in hepatic stellate cells of liver; the activation of these cells is a key event in liver fibrogenesis.

9.7.43.2 Regulation

Some of the research on regulation overlaps that presented for the *CYP11B1* gene (*vide supra*). A CRE/Ad1 element and ATF-1 (and ATF–2?) play roles with both the *CYP11B1* and *CYP11B2* genes [2060]. However, SF-1 does not appear to regulate P450 11B2, in contrast with *CYP11B1* [1988]. Many aspects of regulation remain to be further investigated, including the mechanisms of the observed Ca^{2+} and cyclic AMP signaling [2061] and the effects of kinase inhibitors [2062, 2063].

Transforming growth factor (TGF) β_1 inhibits aldosterone production in human adrenocortical cells by inhibiting P450 11B2 expression [2064]. P450 11B2 expression (in a human adrenocortical cell line) was increased by the orphan nuclear receptors NURR1 and NGF1B [1989]. Levels of NURR1 and NGF1B were strongly induced by angiotensin II, the major regulator of human P450 11B2 expression in vivo. The NBRE-1, Ad5, and Ad1/CRE *cis* elements were all concluded to be involved in both basal and angiotensin-stimulated transcription of human P450 11B2 [1989].

The protein kinase C ligand 12-*O*-tetradecanoy-pharbol-13-acetate (TPA) has been reported to inhibit angiotensin II-stimulated P450 11B2 gene expression (in a H295R human adrenocortical cell line) [2065]. TPA was concluded to inhibit the angiotensin II-dependent activation of P450 11B2 transcription via the p44/42 mitogen-activated protein kinase (MAPK) signaling pathway, leading to an increase in the level of nuclear JunB [2065]. In addition, protein kinase C–E inhibits P450 11B2 gene expression through the ERK/1 signaling pathway (and Jun B) [2066].

Calcineurin mediates angiotensin II-induced upregulation of P450 11B2 transcription [2067].

Like P450 11B1, the P450 11B2 gene is regulated by transposable elements and conserved *cis* elements [2068]. The promoter contains two Alu elements imbedded in a truncated L1 element, breaking up L1 into three fragments. Alu functions as an enhancer in P450 11B2, as in P450 11B1 (*vide supra*). As mentioned earlier, Ad5 and SF-1 binding elements in the proximal core promoter are important in transcription [2068].

Polychlorinated biphenyls have been reported to upregulate P450 11B2 [2069, 2070], apparently via increasing mRNA stability by an unknown mechanism [1992]. However, concentrations < 10 μM were not used, and the relevance of these findings to health is unclear.

9.7.43.3 Genetic Variation

As in the case of the *CYP11B1* gene, many *CYP11B2* variants have now been defined from clinical studies. For review, see [2071]. The many variants [2072, 2073] have been related to a number of diseases, including congenital hypoaldosterism [2074], salt-wasting syndrome [2075], adenoma [2076, 2077], treatment for diabetic nephropathy [2078], high-altitude pulmonary edema [2079, 2080], metabolic syndrome [2081], hypertension [2082–2095], stroke [2096], atrial fibrillation [2097], and other cardiovascular risks [2098].

The "crossovers" between P450s 11B1 and 11B2 yield inactive P450 11B2, as well as P450 11B1 [2016, 2017, 2099, 2100]. Other variants in *CYP11B2* were associated with corticosterone methyloxidase I and II deficiency [2055, 2056,

2101]. Variants in *CYP11B2* have also been linked to idiopathic hyperaldosteronism, a condition characterized by autonomous production of aldosterone and arterial hypertension [2102]. A variant in the promoter region of *CYP11B2* (−344 TK) has been associated with predisposition to essential hypertension [2103].

9.7.43.4 Substrates and Reactions

P450 11B2 catalyzes the three-step conversion of 11-deoxycorticosterone to aldosterone, with 11β-hydroxylation, 18-hydroxylation, and 2-electron oxidation of the 18-carbinol (Figs. 9.12 and 9.23). No other substrates are known. Information about the processivity of the human enzyme (i.e., extent of release of intermediate products) is not available at this time.

Strushkevich et al. [2019] have presented evidence arguing the three-step oxidation of deoxycorticosterone to aldosterone (Figs. 9.23b and 9.24) is a processive one, in that 11β-corticosterone was not oxidized to the product. However, the question has not been analyzed in the usual ways of addressing these questions, e.g., with time course and pulse-chase experiments.

Another recent development is the oxidation of the nonclassical substrate methandienone by P450 11B2 [2104] (Fig. 9.25). The 11β- and 20β-hydroxynorsteroids were formed. Thus, the catalytic selectivity of this steroid hydroxylase may be more relaxed than previously assumed.

9.7.43.5 Structure

In 2013, Strushkevich et al. [2019] published structures of human P450 11B2 with the substrate deoxycorticosterone and an imidazole-based inhibitor, fadrozole. The active site is lined with the same residues as present in P450 11B1 (in that region), and most of the divergent residues apparently associated with the P450 11B2 catalyze activity (18-hydroxylation) are located in the I-helix and loops around the H-helix [2019].

Homology and pharmacophore models have been published [2026, 2073, 2105]

9.7.43.6 Inhibitors

Progress towards clinically useful inhibitors of P450 11B2 has been reviewed recently [2106, 2107]. A number of inhibitors have been produced [2036, 2038, 2108–2114]. These inhibitors are intended for use in congestive heart failure, myocardial fibrosis [2030, 2115–2117], and prostate cancer [2039]. Another intended use is hypertension [2030], and one inhibitor has reached a clinical trial (phase 2) [2118].

9.7.43.7 Clinical Issues

Although there is a rationale for developing inhibitors of P450 11B2 (Sect. 7.43.5, *vide supra*), the major clinical issue is genetic disorders of P450 11B2 insufficiency. Genetic variants and relationship to several diseases, particularly hypertension, have been covered in Sect. 7.43.3 (*vide supra*). In addition, age-related association of variants has been considered in relation to breast cancer risk [2119].

The issues of congenital adrenal hyperplasia and types I and II corticosterone methyloxidase deficiency in individuals with attenuated P450 11B2 activity have already been mentioned. The other issue also mentioned is elevated aldosterone. Several studies have reported an association between variants and essential hypertension, although the measurements of aldosterone excretion are still lacking in some studies [2120]. Other studies show association of the −344C allele with increased left ventricular size [2121–2123]. The hypertension association has been seen in several studies [2082–2095, 2120, 2121, 2124, 2125] but not in a Japanese study [2126].

9.7.44 P450 17A1

17α-Hydroxylation and the 17α,20-lyase reaction ("desmolase") are two important reactions in steroid biosynthesis (Figs. 9.24 and 9.26). Cloning of a cDNA which, when expressed, yielded both activities established the role of what is now known as human P450 17A1 (previously termed P45017$_\alpha$, etc.) [2127]. The gene [2128] showed similarity to *CYP21A1*. The demonstration of both 17α-hydroxylation and 17α,20-lyase catalytic activities in a single protein established work previously done with purified hog protein [2129]. The two activities have long been known

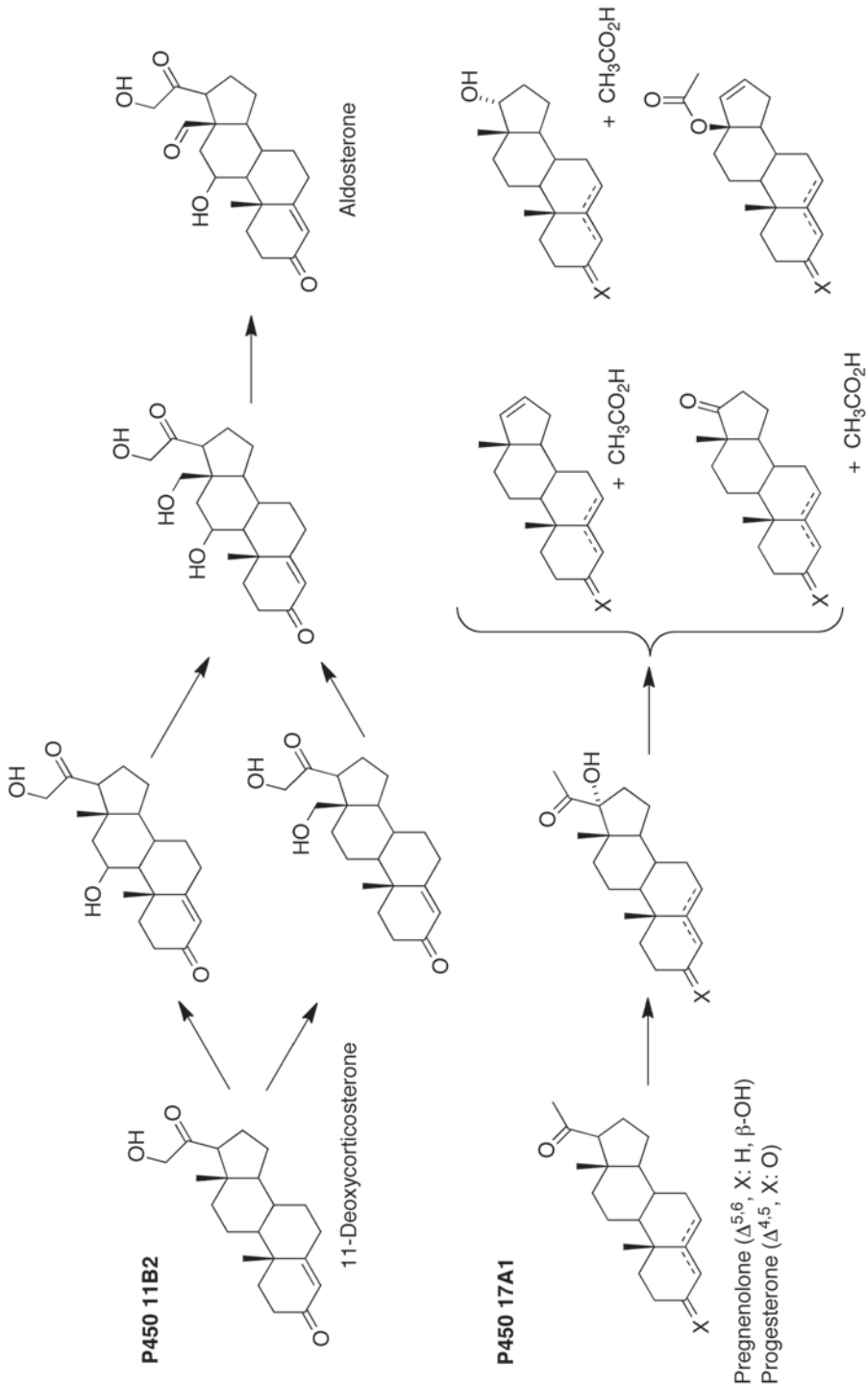

Fig. 9.24 Multistep P450 reactions catalyzed by human P450s 11B2 and 17A1

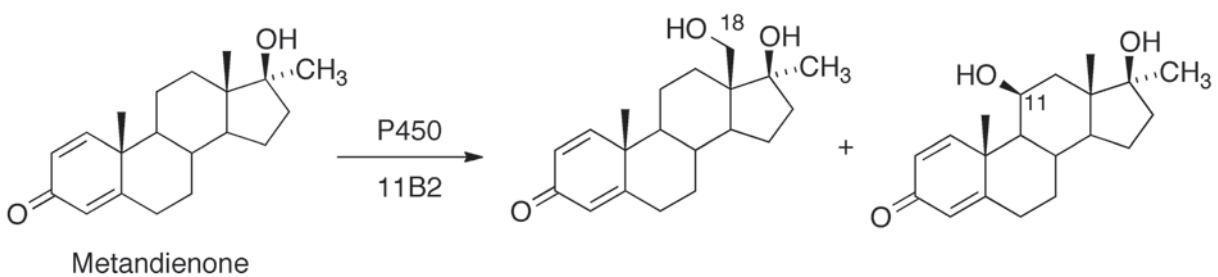

Fig. 9.25 Oxidation of methandienone by P450 11B2

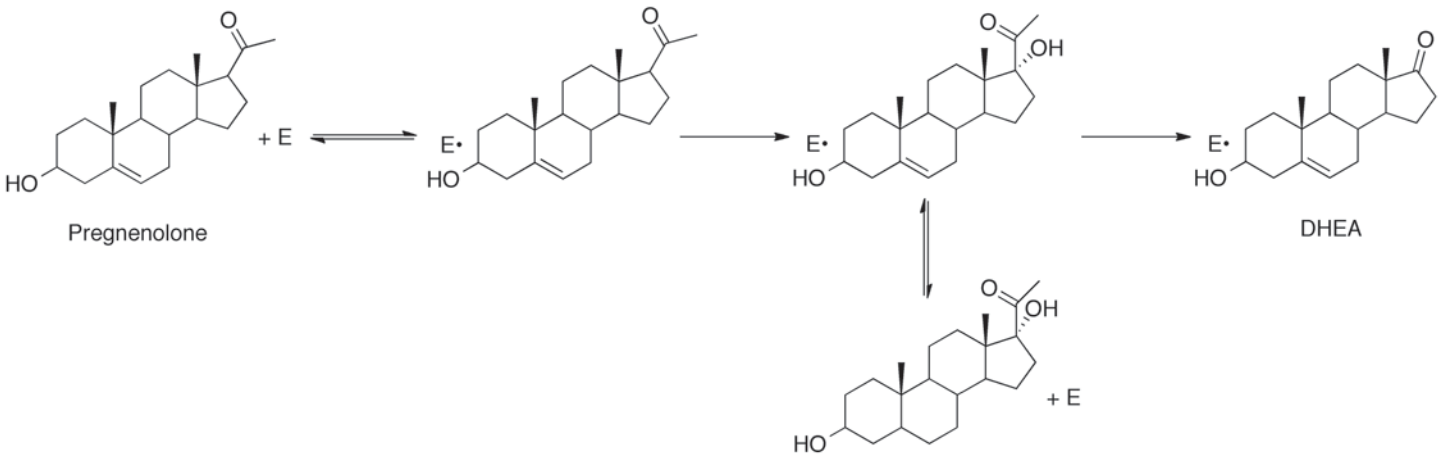

Fig. 9.26 Oxidation of steroids by P450 17A1. P450 17A1 is designated *E* (enzyme). Pregnenolone (shown) and progesterone are oxidized to *DHEA* and androstenedione, respectively, via 17α-hydroxy intermediates. The relative rates of the individual steps, especially the dissociation of the 17α-hydroxy product, determine the processivity of the reaction. In teleost fish, P450 17A2 is also present and catalyzes only the first reaction [2176].

to be regulated by cytochrome b_5 [2130, 2131], and aspects of this duality of function still remain unclear.

9.7.44.1 Sites of Expression

P450 17A1 is a microsomal enzyme (Fig. 9.24, Table 9.2). Human P450 17A1 is expressed in steroidogenic tissues, including adrenals and gonads. The enzyme has also been reported in fetal kidney, thymus, and spleen [2132]. The enzyme has also been found in human (adult) heart [2133] and adipose tissue [2134]. Recently P450 17A1 expression in the human fetal nervous system has been reported [2135].

9.7.44.2 Regulation

As with the other steroidogenic P450s, the regulation of the *CYP17A1* gene is relatively complex. Induction of P450 17A1 has long been known to be cyclic AMP mediated and the induction is suppressed by testosterone (mouse model) [2136], and a cyclic AMP response region was mapped in porcine Leydig cells [2137].

Nuclear factor-1 was implicated in the up-regulation of P450 17A1, acting on the promoter in the cells isolated from patients with PCOS [2138]. Sphingosine was reported to regulate P450 17A1 transcription by binding to SF-1 [2139]. The regulatory protein SMAD3 was reported to inhibit SF-1-dependent activation of the P450 17A1 promoter in human H295R cell culture [2140]. TGFβ inhibited P450 17A1 transcription in the H295R cells via activin receptor-like kinase 5 [2141]. Phosphorylation of CtBP1 by cyclic AMP-dependent protein kinase modulated induction by stimulating partnering of CtBP1 and 2 [2142]. Protein kinase C-induced activin A suppressed P450 17A1 expression [2143].

The homeodomain protein Pbx1 was shown to interact with protein kinase A in the cyclic AMP-dependent regulation (at −250/−241) of the human *CYP17A1* gene [2144]. Further analysis showed interaction at a cyclic AMP-related site (−80/−40) by SF-1 [2145]. Further, interactions were shown for Sp1 and Sp3 (−227/−184) and NF-1C (−107/−85 and −178/−152) [2146]. SF-1 (*vide supra*) also interacts with p54^{nrb}, NonO, and protein-associated splicing factor [2147]. The ACTH/cyclic AMP response is dependent upon phosphatase activity, as well as kinase activity [2148, 2149]. The cyclic AMP-dependent protein kinase enhances transcription via MKP-1 activation, involving phosphorylation of SF-1 [181].

The Miller laboratory has presented evidence that P450 17A1 is phosphorylated and that this has the effect of stimulating only the lyase activity [2150–2152]. In the most recent work, the phosphorylation is attributed to the (Ser/Thr) kinase p38α [2152]. The increase in lyase activity was ~two-fold. The site(s) of phosphorylation is unknown, and no isolation of a phosphorylated protein has been isolated from a tissue.

9.7.44.3 Genetic Variation

At least 49 different variants have been identified in P450 17A1 from clinical studies [2153]. These will not be reiterated here; some references to roles in individual diseases are presented in Sect. 7.44.7 (*vide infra*). See also Chap. 10 [145].

9.7.44.4 Substrates and Reactions

The generally accepted reactions of P450 17A1 are the 17α-hydroxylation of pregnenolone to 17α-hydroxypregnenolone and of progesterone to 17α-hydroxyprogesterone. 17α-Hydroxypregnenolone is also oxidized to DHEA, and 17α-hydroxyprogesterone is oxidized to androstenedione in the 17,20-lyase reaction (Figs. 9.12, 9.24, and 9.26) [2154, 2155]. The mechanism of the lyase reaction is not completely established, but mechanisms have been proposed using analogs [2156]. Lieberman [2157] proposed alternative reactions, although the suggested pathway involves what would be a very unstable diradical. No other substrates are known presently, other than pregnenolone and progesterone and possibly closely related analogues. Soucy et al. [2158] have provided evidence that human P450 17A1 also converts pregnenolone into 5,16-androstadien-3β-ol, a "16-ene synthase" reaction (without intermediate formation of an alcohol).

The lyase reaction is more prominent in adult adrenals with the Δ^5 steroids (than Δ^4; i.e., with 17α-hydroxypregnenolone than 17α-

hydroxyprogesterone), and this also applies in (human) fetal testis [2159]. P450 17A1 also has trace 21-hydroxylation activity [2160], and the mutation A105 L yields a protein with some 16α-hydroxylation activity [2160, 2161].

A kinetic deuterium isotope of ~4 was observed for the 17α-hydroxylation reaction [2160]. The mechanism of this hydroxylation is presumed to be relatively straightforward "compound I"-type hydroxylation, with C–H bond breaking being at least partially rate limiting. Rates of individual steps in the reaction have not been reported.

The second reaction, the 17,20-lyase reaction, is more complex and difficult to rationalize with a classic compound I mechanism. Work from Akhtar's laboratory led to the proposal that the reaction involves a nucleophilic attack of the ferric peroxide (anion; FeO_2^+, or $Fe^{II}O_2^-$) on the C-20 carbonyl (of 17α-hydroxyprogesterone or pregnenolone) [2156, 2162]. One of the key pieces of evidence was the result of $^{18}O_2$ labeling experiments [2156]. The results of site-directed mutagenesis studies on Thr-306 are also consistent with the conclusions about $Fe^{II}O_2$ involvement [2156, 2163]. Sligar's group has also presented resonance Raman spectra [2164] and solvent deuterium kinetic isotope effect studies [2165] in support of the involvement of this entity.

Further work on the differential effects of cytochrome b_5 on individual catalytic activities has been reported [2166]. The ratio of cytochrome b_5 to P450 is high in testis and this phenomenon might regulate the two activities of P450 17A1. Miller's group has proposed that phosphorylation of Ser and Thr residues in P450 17A1 may alternatively influence the two activities [2152, 2167, 2168].

A second cytochrome b_5 gene has been identified recently and this protein also has the same stimulatory effect on lyase activity [2169]. Auchus et al. [2170] also demonstrated that the same stimulatory effect of cytochrome b_5 could be obtained with apo-cytochrome b_5, arguing against the requirement for electron transfer. P450 17A1 enzymes from other species vary in their ability to catalyze the 17,20-lyase reaction, and comparisons of the rat and human enzymes also led to the conclusion that selective enhancement of the lyase reaction was not due to changes in electron transfer [2171].

The concertedness of the P450 17A1 17,20-lyase reaction has been examined, and two studies both reached the conclusion that much of the 17α-hydroxypregenolone dissociates [2172, 2173]. In one of the studies [2172], the authors concluded that the off-rate was an important factor in determining the balance between 17α-hydroxypregnenolone and DHEA with the beef enzyme. Exactly how cytochrome b_5 would control this rate, which was modeled to be rather slow (2.6–29 min^{-1}), is unclear unless the effect is on the protein conformation.

Studies with human P450 17A1 in this laboratory show that the human P450 17A1 enzyme is relatively distributive for the two reactions, 17α-hydroxylation and the 17,20-lyase cleavage. This was shown using pulse-chase experiments with ^{14}C progesterone or pregnenolone and then adding varying amounts of unlabeled 17α-hydroxy steroid, measuring the attenuation of radiolabel incorporated into the final product. However, the reaction shows more processivity with pregnenolone than progesterone. Further evidence for the distributive nature of the enzyme comes from studies with the inhibitor orteronel (TAK-700), which preferentially inhibits the lyase (second reaction) [2174]. If the enzyme were totally processive, this result would be impossible.

Teleost fish P450 17A1 enzymes catalyze both reactions, similar to human P450 17A1 [2175]. That enzyme is also distributive, more so with progesterone than pregnenolone [2176]. The related fish P450 17A2 catalyzes only the 17α-hydroxylation, with or without cytochrome b_5 [2175, 2176]. Cytochrome b_5 did not affect the processivity of the fish P450 17A1 reactions [2176].

Another issue already mentioned (Sect. 7.44.2) is phosphorylation, which has been reported to favor the second reaction (lyase) [2152]. This result is a bit of an enigma in that cytochrome b_5 is considered to have an anionic region ("patch") that binds to basic residues in P450 17A1, as evidenced by site-directed mutagenesis [2177].

A phosphate group (in this region?) would add a negative charge and tend to prevent interaction with cytochrome b_5.

Another enigma about P450 17A1 catalysis is raised from the results of Scott and her associates [2178], who presented NMR evidence that cytochrome b_5 and NADPH-P450 reductase bind to the same section of P450 17A1 and therefore compete for binding. Strong evidence has been presented that electron transfer is not involved in the stimulation of P450 17A1 by cytochrome b_5 [2170]. Therefore, both of the electrons used in the P450 17A1 reaction (regardless of whether it is a compound I or ferric peroxide mechanism) must come from the reductase. Thus, the P450 must be reduced to the ferrous state, bind O_2, accept another electron, and thus be in the FeO_2^+ state before the reductase dissociates. This must happen rapidly, and the formal FeO_2^+ entity must be stable enough to persist until the cytochrome b_5 is bound and apparently "allosterically" perturbs P450 17A1 FeO_2^+ to catalyze the lyase reaction. The reaction has a k_{cat} of ~1 min^{-1}, so this must happen in seconds during every catalytic cycle (and the cytochrome b_5 must leave again for NADPH-P450 reductase to begin and reinitiate catalysis). Exactly what occurs will require further study.

What step is rate limiting in the lyase reaction is presently unknown. That reaction seems impervious to the use of kinetic isotope effects to study the nature of C–C bond cleavage (unless ^{13}C isotope effects could be used).

9.7.44.5 Structures

Much of the information about the significance of active site residues comes from analysis of mutations in patients presenting with diseases (see Sect. 7.44.2, *vide supra*). The changes H373 L and P409R [2179] led to a loss of heme incorporation. Mutation at Thr-306, possibly involved in protonation of Fe–OO^- or O–O cleavage, impaired 17α-hydroxylation more than the lyase reaction [2180]. However, the change R346A selectively abolished lyase activity [2181], as did F417C [2182]. Mutations at Lys-83, Arg-347, Arg-358, and Arg-449 produced proteins that were refractory to cytochrome b_5 stimulation and attenuated in lyase activity [2183–2185]. Of these, only R347H and R358Q have been found in patients [2186]. Some variants found in patients do cause the loss of both 17α-hydroxylation and the lyase reaction, however [2187, 2188].

Some animal P450 17A1 enzymes have different ratios of 17-hydroxylation/lyase activities, and efforts have been made to use these properties to define more elements controlling the latter steps, although the results have been limited [2189, 2190].

A number of additional homology models have been published [2024, 2191–2197].

In 2012, DeVore and Scott [2153] published an X-ray crystal structure of human P450 17A1 bound to the inhibitors abiraterone and TOK-001 (Sect. 7.44.6, *vide infra*). As might be anticipated, the pyridine nitrogen is bound to the heme iron. The binding mode was considered to be different than observed for a number of other P450s that use steroids as substrates. This structure may be useful in rationalizing the variants seen in clinical problems.

As discussed in Sect. 7.44.4 (*vide supra*), one of the mechanistic curiosities is the interaction of cytochrome b_5 with P450 17A1, which (in part) regulates the balance between the 17α-hydroxy and 17,20-lyase products. An NMR study with cytochrome b_5 led to the conclusion that the protein occupies a position at a site on P450 17A1, including Arg-347, Arg-358, and Arg-449 [2178]. The same site is believed to be occupied by NADPH-P450 reductase.

The dual nature of the P450 17A1 in catalyzing sequential reactions can be addressed using fish orthologs. Teleost fish have two P450 17A genes, 17A1 and 17A2 [2175]. Fish P450 17A1 resembles mammalian P450 17A1 in catalyzing both the 17α-hydroxylation and the lyase reactions, but (fish) P450 17A2 only catalyzes the 17α-hydroxylation [2175]. Fish or human cytochrome b_5 stimulates only the lyase activity. This laboratory, collaborating with Prof. Martin Egli, has crystallized both zebra fish P450 17A1 and 17A2, with abiraterone bound to each and progesterone bound to P450 17A2 [2176], as in the human P450 17A1 structure [2153].

9.7.44.6 Inhibitors

Inhibitors of P450 17A1 have been studied for some time. Interestingly, ketoconazole inhibits lyase activity but not 17α-hydroxylation activity [2198]. 7α-Thiospirolactone is a mechanism-based inhibitor of (guinea pig) P450 17A1 [2199].

A number of steroidal inhibitors have been studied, primarily with the goal of treating cancers [2200–2202] [2203, 2204]. The enantiomer of progesterone (*ent*-progesterone) is reported to be a competitive inhibitor of P450 17A1 (K_I 0.2 μM) [2205].

Nonsteroidal inhibitors have also been studied [2206, 2207].

Molecular modeling (Sect. 7.44.5) has also been applied to searches for inhibitors [2197, 2208]. Other approaches utilize P450 17A1 expressed in *E. coli* to screen for P450 17A1 inhibition in medium- to high-throughput systems [2209, 2210].

One interest in inhibition of P450 17A1 is treating prostate cancer. The concept is that prostate cancer is stimulated by androgens, and the goal is to block production of androstenedione (from progesterone/17α-hydroxyprogesterone). This is a particular issue in "castration-resistant" prostate cancer.

A number of inhibitors have been published [2035, 2037, 2211–2215]. For reviews see [2216–2219]. Abiraterone is a leading inhibitor, currently approved for use for prostate cancer [2220–2225]. Another drug in clinical trials is orteronel (TAK-700), which shows selective inhibition of the lyase reaction [2174, 2226]. The concept is to block androgen production (i.e., androstenedione formation) and maintain production of other steroids for normal physiology.

9.7.44.7 Clinical Issues

P450 17A1 has a central role in human steroid metabolism because of its role in regulating steroid flux (Fig. 9.12). There are two dominant clinical issues with P450 17A1. One is various diseases associated with hormone imbalance. P450 17A1 is at a branch point and involved in production of glucocorticoids and sex hormones (androgens and estrogens), and therefore a variety of maladies can be associated with changes. The other issue, addressed under Sect. 7.44.6 (*vide supra*), is the use of P450 17A1 inhibitors (especially lyase inhibitors) to treat androgen-stimulated tumors. The second point will not be treated further here.

The clinical issues for which research has been done to implicate associations with P450 17A1 status (usually genetic) include endometrial cancer [2227], prostate cancer [2228 2003, 53455, 2229], breast cancer [852, 2230], endometrial cancer [2231], non-Hodgkin's lymphoma [2232], infertility [2233], pregnancy loss [2234], early embryonic lethality [2235], short menstrual cycles/early contraceptive use/BRCA mutations [2236], secondary amenorrhea [2237], PCOS [2238], endometriosis [2239], and acne [2240]. Perturbations in P450 17A1 lead to problems in adrenarche, aging, and PCOS [2155, 2241]. Some of the more serious variants have been mentioned already. Another variant is related to a case of pseudohermaphroditism involving lack of lyase activity [2242].

Some of the other possible disease conditions or risks are being studied in relationship to less serious variants. In most of these cases, the relationships are more difficult to establish than in the serious diseases. A possible link of *CYP17A1* deficiency has been made with rheumatoid arthritis [2243]. Little influence of genetic variation was seen on age of menarche [2244]. However, a link was made between a particular variant and the prediction to use hormone replacement therapy (i.e., postmenopausal estrogen therapy) [2245]. No association was found with PCOS in a study with an SNV at the regulatory Sp1 site [2246].

Much attention has been given to the possibility of a link between *CYP17A1* allelic SNVs and breast cancer risk [2247]. The epidemiology results are mixed at best [2248–2251], and a conclusion in favor of a relationship cannot be made at this time [2230, 2252, 2253].

As with some other P450s, circulating antibodies to P450 17A1 are seen in some autoimmune diseases, e.g., autoimmune polyglandular syndrome and Addison's disease [1969, 2254], but no causal relationship has been demonstrated.

9.7.45 P450 19A1

P450 19A1 is the classic "aromatase," often known by that name in endocrinology. This enzyme oxidizes the androgens (e.g., androstendione and testosterone) to estrogens (estrone and 17β-estradiol, respectively) (Fig. 9.22). This process is very important in normal physiology and also a target for inhibition in some tumors.

9.7.45.1 Sites of Expression

Estrogens have a number of functions, not only in feminization. Although estrogens are often considered "female" hormones, they are also important in males (e.g., see material regarding brain, *vide infra*). P450 19A1 is even found in the penis [2255] and is important in male reproduction [2255, 2256]. Sites of (human) expression include the ovaries and testes, placenta and fetal (but not adult) liver, adipose tissue, chondrocytes and osteoblasts of bone, vasculature smooth muscle, and several sites in brain, including parts of the hypothalamus, limbic system, and cerebral cortex [2257]. As discussed later, regulatory mechanisms differ considerably in these tissues. P450 19A1 is also expressed in some tumors, particularly those derived from these tissues.

Evidence for P450 19A1 in the brain has been reported [2258], and a mouse *CYP19A1* knockout provides evidence that estrogens are required for brain development [2259]. The actions of androgens and estrogens in the gonadal tissues are fairly well understood but less is known in the brain. Androgens and androgen-derived estrogens regulate complementary and interacting genes in many neural networks [2260]. P450 19A1 expression in skeletal muscle has been reported [2261].

Although P450 19A1 is generally considered an extrahepatic P450, there is evidence for some expression in human liver [2262]. P450 19A1 peptides have been detected in liver microsomes (treated with trypsin) by LC–MS [635]. Oxidations of dihydrotestosterone attributable to P450 19A1 have been observed in human liver microsomes [1373].

Evidence has been presented that P450 19A1 dimers exist in membranes and that P450 19A1 does not dimerize with P450 17A1 [2263].

9.7.45.2 Regulation

The regulation of the *CYP19A1* gene is quite complex, primarily because of the use of four tissue-selective promoters [2257, 2264]. The promoters have been reviewed [2265]. Much of the research has been in the area of cancer. Either the I.l, I.4, I.f, and I.6 sequence is read as exon I and spliced in to the mRNA, depending upon the tissue. However, exon I does not code for the protein, so the P450 19A1 enzyme is always the same.

In preovulatory follicles and corpora lutea of human ovary, the 5′-untranslated region of P450 19A1 transcripts is encoded by exon IIa [2266]. The major operatives here are CRE and SF-1 elements [2257].

In adipose tissue, the promoter from exon I.4 is utilized [2257]. The same exon is utilized in bone and skin [2257] and in leiomyoma tissue derived in myometrium [2267]. This system is regulated with Sp1, a glucocorticoid regulatory element, STAT3, and possibly PPARγ [2257, 2268]. Preadipocytes also involve regulation with LRH-1 [2269].

In placenta exon I.1, an 89-kb upstream element is utilized [2257]. This is a strong promoter and involves C/EBP-β [2257]. A strong positive enhancer element between −42 and −501 is present [2270]. The possibility exists that VDR/RXRα heterodimers and PPARγ may have effects [2257].

Regulation in bone uses exon I.6 [2257]. The study of regulation in bone is less extensive than in other sites, and 1,25-dihydroxycholecalciferol, interleukins, TNFα, and TGF-β_1 have stimulatory activity.

Regulation in brain uses exon I.f and has also not been as extensively studied [2257]. P450 19A1 does seem to be upregulated by androgens.

Regulation in fetal liver involves exon I.4, as with adipose tissue [2257]. The same pattern appears to apply in skin fibroblasts and intestine.

In cancer cells, alternate regulatory pathways are utilized [2257]. EP2 and EP4 receptors regulate P450 19A1 expression in human adipocytes and in breast cancer cells, involving BRCA1-p300 exchange [2271]. CCAAT/EBPβ upregulated promoters I.2/II in breast cancer epithelial

cells [2272]. P450 19A1 transcription is also enhanced by RXRα/RORα in breast cancer cells [2273, 2274]. In skeletal muscle, the P450 19A1 gene is a target of the factor Runx2 [2275]. In human ovarian granulosa cell-like KGN cells, activin stimulates P450 19A1 gene expression via the Smad2 signaling pathway [2276]. In granulosa cell tumors, P450 19A1 is a direct target of FOXL2 to C134W via a single highly conserved binding site in the ovarian-specific promoter [2277]. In human placental syncytiotrophoblasts, cortisol induces P450 19A1 expression through the cyclic AMP/Sp1 pathway [2278].

A vitamin D analog inhibits P450 19A1 expression by dissociation of the comodulator Williams syndrome transcription factor (WSTF) from the promoter [2279]. PPARγ agonists downregulate P450 19A1, via BRCA1 and prostaglandin E2 [2280, 2281]. TCDD has been reported to induce P450 19A1 in human glioma cells [2282].

In the area of post-transcriptional regulation, the alternative miscoding exons 1 are involved [2283]. Some evidence for control of genes by DNA methylation has also been reported [2284, 2285].

9.7.45.3 Genetic Variation

The cypalleles website (http://www.cypalleles.ki.se) shows only five allelic variants of the human CYP19A1 gene, which seems surprising compared to some of the other steroidogenic P450s, e.g., P450s 17A1 and 21A2 (Sects. 7.44 and 7.46, respectively). These have been studied with regard to breast cancer but without convincing relationships (*vide infra*); also, there was no relationship with breast density [2286].

Relatively few cases of aromatase deficiency have been reported [2257, 2287], and some of the clinical cases may be the result of NADPH-P450 reductase deficiency. In vitro steady-state kinetic analysis of one variant (T201M) has been found it to be more active than the *1 (wild type) P450 19A1 [2288]. See Sect. 7.45.7 for clinical issues related to genetic abnormalities.

9.7.45.4 Substrates and Reactions

The reaction involves three steps and has been the subject of considerable mechanistic interest (Fig. 9.22). Androstenedione is converted to estrone, testosterone to 17β-estradiol, and 16α-hydroxytestosterone to estriol. The first two steps are relatively straightforward, e.g., $RCH_3 \rightarrow RCH_2OH \rightarrow CHO$ (at C19). The third step was difficult to rationalize with "classic" FeO^{3+} chemistry, and there has been general acceptance of a FeO_2- based mechanism originally developed by Akhtar [2289, 2290] and Robinson [2291] and further studied in models by Coon and Vaz [2292].

The possibility of utilization of DHEA as a substrate for estrone synthesis has been proposed but not addressed directly [2293].

P450 19A1 also catalyzes oxidation reactions with related compounds, some of which may have physiological relevance (Fig. 9.27). 3-Deoxy androgens are oxidized (19-methyl deformylation) in a similar manner [2294]. Recently this laboratory has demonstrated that androstenedione and testosterone are oxidized to the 19-formic acid derivatives by 2-electron oxidation of the 19-aldehyde [2295]. This product (previously reported as an androstenedione derivative in porcine granulosa cells [2296]) apparently is stable and is not oxidized to an estrogen (it is sensitive to acid-catalyzed decarboxylation, which yields 19-nor-androgens). We also found that the three porcine P450 19A1 enzymes all make as much of the 19-formic acid product as estrogen, from either testosterone or androstenedione. The in vivo relevance remains to be established. The product estrone is known to be further oxidized (slowly) by 2-hydroxylation [2297, 2298].

Dihydrotestosterone, a more potent physiological androgen, is also oxidized by P450 19A1 in the same general way as the other androgens [1373] (Fig. 9.27). The products are deformylated and one is further oxidized (2-hydroxylation), but estrogens are not formed [1373]. Whether the 19-aldehyde forms the 19-carboxylic acid is not known.

The three-step reaction has been shown to be mainly distributive [220]. The reaction can be initiated with any of the intermediate steroids (Fig. 9.22; yielding the final estrogenic product). Pulse-chase experiments show the distributive nature of the products, and a reaction with a limited amount of androstenedione shows a smooth progression through the two intermediate products [220].

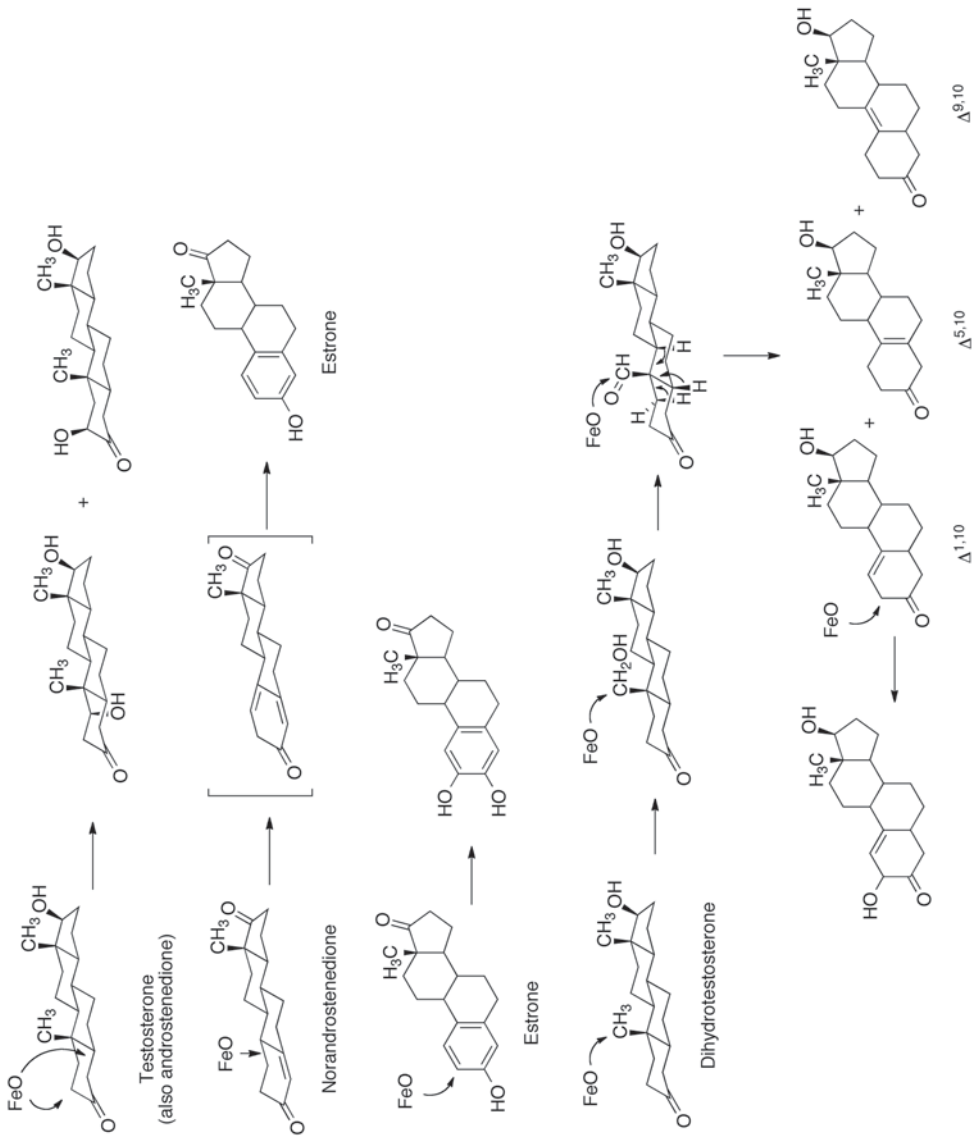

Fig. 9.27 Known reactions of human P450 19A1 (in addition to aromatization of androgens) [1373, 2300]

Exactly which catalytic step is rate limiting within each of the three reaction steps is not clear. With placental microsomes as the enzyme source, an intermolecular kinetic deuterium isotope effect of 3.2 was reported for the first step and no kinetic isotope effect for the second step [2299]. An even higher kinetic hydrogen isotope effect was estimated by Osawa et al. [2300]. Sligar and his associates reported spectroscopic studies on the $Fe^{2+}O_2$ form of the enzyme in the presence of androstenedione, with a decomposition rate of 0.7 s^{-1} (42 min^{-1}) at 37 °C [2301] which is roughly equivalent to the rate constant in a model for the first step [220]. Sligar's group has also presented electron paramagnetic resonance (EPR) spectral evidence for the formation of an FeO_2^+ ($Fe^{II}O_2^-$) intermediate, formed by cryoradiolysis (at 77 K), using EPR detection [2302]. The relevance to catalysis has not been investigated (i.e., product formation was not measured).

The first two oxidations in the conversion of androgens to estrogens are relatively straightforward and can be readily rationalized with classic compound I mechanisms, i.e., hydroxylation of a methyl group and the oxidation of a carbinol to a *gem*-diol/aldehyde. The third step has been problematic and has invited a number of proposals over the years, including (A-ring) 1-hydroxylation, 2-hydroxylation, 4,5-epoxidation, and hydrogen abstraction from C-19 followed by rearrangement [2289, 2291, 2299, 2303–2317]. Pathways to estrogens (and formic acid) can be drawn in these cases, but they have been ruled out for one reason or another, e.g., ^{18}O labeling results for the 2-hydroxy mechanism [2315]. Currently the most widely accepted mechanism is probably the ferric peroxide mechanism proposed by Akhtar and supported by some $^{18}O_2$ labeling results [2305]. An alternate mechanism proposed by Hackett et al. [2308] involves compound I hydrogen abstraction of H-1β [2303, 2304] followed by an electronic rearrangement and a "concerted" C–C bond scission without formal hydroxylation. This is an adaptation of a mechanism proposed by Covey et al. earlier, which begins with hydrogen atom abstraction from the C-19 *gem*-diol [2312].

The mechanism has involved considerable debate over the years [2289, 2304, 2305, 2315]. A number of approaches can be applied to the mechanistic question, including studies with simplified models [2291, 2318–2321], synthesis of potential intermediates for testing with the enzyme [2303, 2304], application of theory [2308, 2309], spectroscopy [2301, 2302], and isotopic labeling studies [2299, 2305].

Recent work in this laboratory bears on the mechanism [2295]. As mentioned earlier, purified recombinant P450 19A1 converts 19-aldehyde androgens (and 19-methyl androgens) to the 19-formic acid derivatives (Fig. 9.28). The products appear to be relatively stable (formation of estrogens requires base-catalyzed release of the carbon as CO_2). These findings indicate that P450 19A1 compound I is capable of being formed and used in the last step.

When either 19-deuterated 19-oxoandrostenedione or testosterone was incubated with recombinant P450 19A1 and $^{18}O_2$, ^{18}O label was *not* incorporated into the recovered formic acid (Fig. 9.28). These results differ from those reported previously [2289, 2305]; the major technical differences are the use of recombinant purified P450 19A1, a more sensitive probe for trapping and analyzing formic acid, and the use of UPLC-coupled high-resolution mass spectrometry, which avoided issues inherent in analysis of labeled formic acid [2295]. The results are not consistent with the proposed ferric peroxide mechanism, in which an ^{18}O atom is expected to be recovered in formic acid.

An issue with the ferric peroxide mechanism is that the substrate is in the (hydrated) *gem*-diol form following the second reaction (Fig. 9.28). However, the proposed ferric peroxide mechanism involves a nucleophilic attack ($Fe^{2+}O_2^-$) on the carbonyl (aldehyde). Thus, the *gem*-diol must be dehydrated before this step can run. The rate of dehydration has been estimated at > 0.5 s^{-1} (in the absence of P450 19A1 using ^{18}O exchange methods [2295], which is faster than the k_{cat} (8 min^{-1}) for going from 19-hydroxy androstenedione to estrone [220]. The reaction could occur with the aldehyde or the *gem*-diol, the latter of

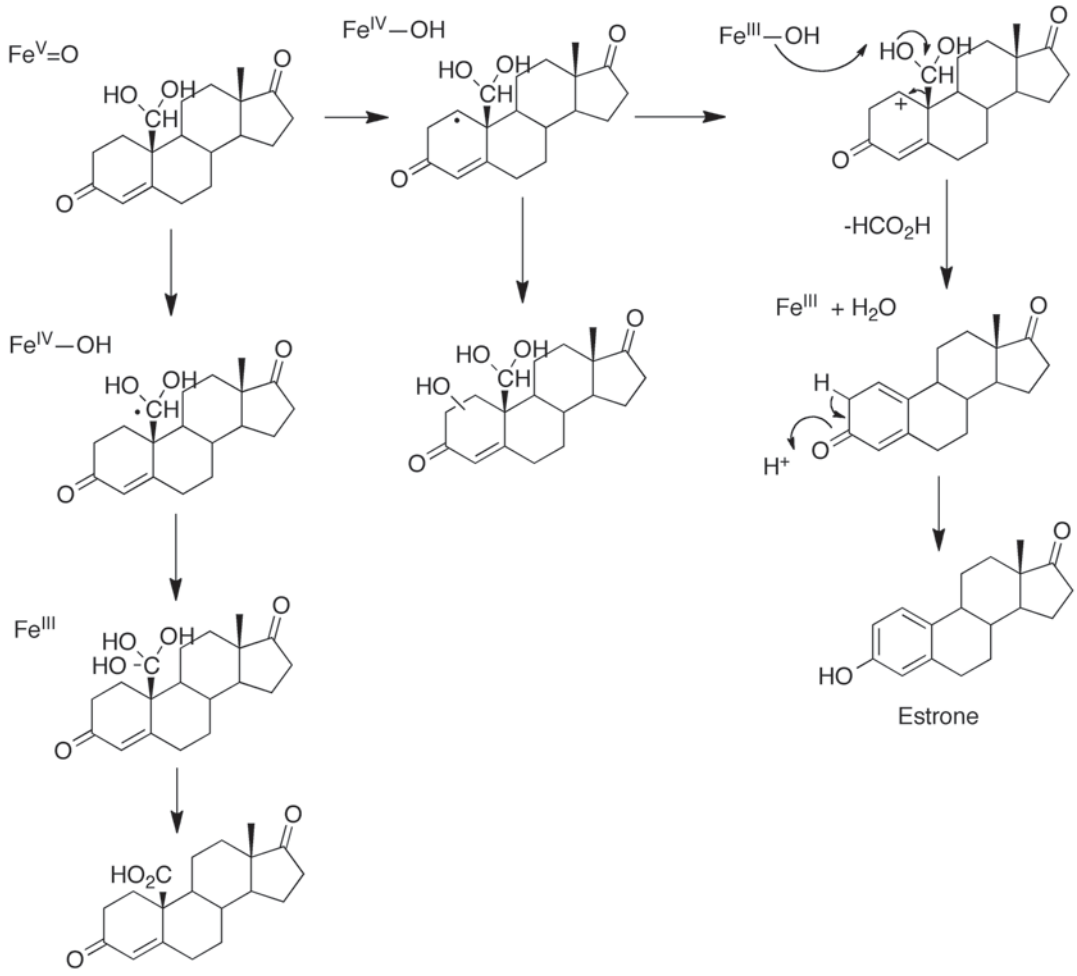

Fig. 9.28 Unified mechanism of C–C cleavage in the third oxidation step of androgen conversion to estrogens by P450 19A1, including the formation of androgen 19-carboxylic acids. The mechanism is based on labeling studies with $^{18}O_2$ [2295]

which is more consistent with the proposals of Covey et al. [2312] and Hackett et al. [2308].

It is of interest to note that hogs have three P450 19A1 genes, and one of these converts testosterone to 1β-hydroxytestosterone [2322]. Both androstenedione and testosterone are converted to some 1β- and 2β-hydroxy products by purified human P450 19A1 [2300], indicating that the FeO^{3+} complex can position itself to abstract an H-1 or H-2 atom from the androgen substrate.

9.7.45.5 Structure

One of the historic problems in studying structure–function relationships in P450 19A1 has been the availability of expression systems. Recently several *E. coli* systems have been developed [220, 2323–2325].

Some homology modeling studies have appeared [2326, 2327]. In 2009, Ghosh et al. [2328] published a crystal structure of P450 19A1 purified from human placental samples, without any modification (even at the N terminus), the only mammalian P450 to be crystallized in such a way. The structure contains a bound androstenedione substrate, positioned in a manner to make oxidation feasible. The structure has been utilized in the design of new inhibitors [2329].

More recently, recombinant (*E. coli*) P450 19A1 has been expressed and crystallized, along with some active site mutants [2325].

9.7.45.6 Inhibitors

The literature on clinical use of aromatase inhibitors for cancer treatment is immense, and much has been published since the last version of this chapter [149]. The topic has been reviewed many times [2330–2333], including some reviews by A. Brodie, a pioneer in this area [2334, 2335]. Breast cancer is probably the major target area for P450 19A1 inhibition, but other cancers are also under investigation [2336].

Today the process has reached the stage of "third-generation" inhibitors [2337], moving beyond early drugs such as aminoglutethimide [2338]. The newer inhibitors are more effective in lowering the body load of estrogens [2339]. One example of a newer drug is exemestane, a site-directed Michael acceptor (compared with the ER antagonist tamoxifen) [2338–2341].

The leading P450 19A1 inhibitors in use today are primarily (but not exclusively, Sect. 7.45.7, *vide infra*) exemestane, anastrozole, and letrozole [2342, 2343]. Although the potency of these three inhibitors is excellent, efforts to develop new inhibitors are continuing using other chemical approaches [2329, 2344–2349].

These inhibitors are not without toxicities [2350], although most of the expected issues can be anticipated due to generalized attenuation of estrogen levels throughout the body. Other inhibitory Michael agents have been prepared from prostaglandin J_2, but detailed characterization has not been done [2351]. Other nonsteroidal inhibitors of P450 19A1 are also under consideration [2352].

The point has been made by Simpson et al. [2257] that a future goal of P450 19A1 inhibition should be tissue selectivity. The diverse role of P450 19A1 in different tissues might indicate that generalized inhibition of estrogen synthesis may be less than desirable. Targeted inhibition of P450 19A1 could, in principle, be achieved by (1) selective targeting of inhibitors of P450 19A1 catalysis to tumors/individual organs or (2) targeted downregulation of P450 19A1 synthesis in selected areas.

Finally, *CYP19A1* genetic variants have been considered in relationship to breast cancer patient response to inhibitors [2353].

9.7.45.7 Clinical Issues

The two major clinical issues with P450 19A1 are (1) disease states associated with genetic variations and (2) use of aromatase inhibitors to block estrogen-dependent diseases [2354]. Serious cases of congenital aromatase deficiency in adults appear to be relatively rare [2257, 2355, 2356] and have been treated with estrogen replacement therapy [2357]. However, some children are considered to have attenuated P450 19A1 activity [2358]. Studies with P450 19a1-knockout mice show expected reproductive and sexual phenotypes and also adipose and bone phenotypes [2355, 2359], as well as a sociosexual behavior phenotype [2360]. There are known gain-of-function variants, with some issues [2361].

For a review of the significance in cancers, see [2362]. There is consideration of the use of inhibitors for breast cancer prevention in high-risk individuals. P450 19A1 inhibitors have been used extensively for breast cancer (see Sect. 8.45.6) [2363], epilepsy [2364], children with short stature [2365], other pediatric disease [2366] endometriosis [2367], male infertility [2368], and induction of ovulation [2369]. P450 19A1 inhibitors have also been reported to cause arthralgia [2370].

A number of studies have been made on the relationship of *CYP19A1* polymorphisms with breast cancer, but the evidence has not shown a change in risk [2286, 2371]. No strong association was seen for endometriosis [2372]. Genetic variation in P450 19A1 has been considered regarding breast cancer [2373–2376], prostate cancer [2377], lung cancer [2378], response to therapy with aromatase inhibitors [2379], estrogen levels and bone structure in older women [2380], bone loss [2381], polycystic ovary disease [2382], age at menarche [2383], essential hypertension [2384, 2385], craving during alcohol withdrawal [2386], obsessive compulsive behavior and Parkinson's disease [2386], testicular disease [2387], pubertal sagittal jaw growth [2388], and reading, speech, and language [2389].

9.7.46 P450 20A1

9.7.46.1 Sites of Expression

P450 20A1 expression has been reported in liver and brain. In brain, expression was noted in substantia nigra, hippocampus, and amygdala [2390].

9.7.46.2 Regulation

To date, no reports on the regulation of P450 20A1 have appeared.

9.7.46.3 Genetic Variation

No reports of polymorphism or other genetic variation of P450 20A1 have appeared.

9.7.46.4 Substrates and Reactions

Attempts to identify substrates with a recombinant P450 20A1 expressed in *E. coli* have been negative [2390]. However, the expression level was low and should be improved.

It is of interest to note that P450 20A1 is unusual and an ortholog even appears in sponges. It is possible that 20A1 has an important physiological function. One could consider this to be the "most orphan" of the orphan P450s (Table 9.1).

9.7.46.5 Structure

No information is yet available.

9.7.46.6 Inhibitors

Obviously, since no catalytic activity has been reported, there are no inhibitors.

9.7.46.7 Clinical Issues

No clinical issues have been considered, in light of the lack of information about function.

9.7.47 P450 21A2

P450 21A2 is the enzyme involved in the 21-hydroxylation of progesterone and 17-hydroxyprogesterone, yielding deoxycorticosterone and 11-deoxycortisol from the two substrates, respectively (Fig. 9.12). The 21-hydroxylation reaction is an important step in the synthesis of glucocorticoids and mineralocorticoids, and deficiencies lead to "salt-wasting syndrome," if not treated, and to congenital adrenal hyperplasia in the worst cases.

9.7.47.1 Sites of Expression

The major site of expression is the adrenal cortex. This reaction has been known for some time, and many of the early biochemical studies were done with beef adrenals because of the need for large amounts of tissue [2391].

Low amounts of P450 21A2 have been reported in human lymphocytes [2392] and brain [2393]. Any specific function in these tissues is unknown at this time.

9.7.47.2 Regulation

The regulation of P450 21A2 has some similarity to that of P450 17A1, in that both are regulated by ACTH. The cyclic AMP-responsive sequence in the 5′-flanking region [2394] uses adrenal-specific protein factor and an Ad4-like sequence [2395]. One issue in the regulation of the *CYP21A2* gene is the neighboring homologous but nonfunctional *CYP21A1* pseudogene, which can compete for transcription factors and other regulatory proteins [2396]. In other work, protein kinases A and C and Ca^{2+} were found to regulate *CYP21A2* gene expression in a human cortical cell line [2397].

Another interesting aspect of the regulation of the *CYP21A2* gene is that it is located very close to the major histocompatibility locus, 2.3-kb downstream from the *C4* gene. Transcriptional regulatory elements for the *CYP21A2* gene lie within intron 35 kb of the *C4* gene [2398].

Evidence for regulation by vitamin D has appeared [2399].

9.7.47.3 Genetic Variation

Steroid 21-hydroxylase deficiency is the most common cause of congenital adrenal hyperplasia, and many variants are now known to be associated with the disease. To date, more than 150 clinical variants have been reported, with >97 consisting of missense variants [42, 2400]. In addition to missense variants, deletions [2401] and copy number variants [2402, 2403] have been reported. Ethnic links of the variations have also

been reported, e.g., [2404]. Variations in the promoter region are also known [2405].

The genetics of P450 21A2 variation have been reviewed recently [2406, 2407]. Many genetic variants are the result of recombination with the related pseudogene [2408, 2409]. Some are in the coding region [2410–2412] and the 5′-flanking region [2413]. The incidence of carriers of congenital adrenal hyperplasia is 1–2% in the population, and many deleterious variants have now been identified [2414–2421].

9.7.47.4 Substrates and Reactions

The primary substrates are progesterone and 17α-hydroxyprogesterone, which are hydroxylated only at the 21-position (Fig. 9.12). A minor activity seen with P450 21A2 is 16α-hydroxylation of progesterone, better revealed by use of the (C-17) deuterated substrate (due to "metabolic switching") [2160]. The rates of 21-hydroxylation of progesterone and 17α-hydroxyprogesterone are among the fastest of all mammalian P450s, with catalytic efficiencies of 10^7 and 2×10^6 M^{-1}/s observed in this laboratory for the wild-type human enzyme [2769].

9.7.47.5 Structure

Homology models have been reported [2024, 2422–2426]. The amount of site-directed mutagenesis has been limited, but the disease has yielded many locations for loss of function because the severity of the disease is (inversely) correlated to the residual 21-hydroxylation activity. Many of the variants could be rationalized in the context of a homology model [2422], although some associated with disease are more subtle (e.g., E380D). A structure of the human P450 21A2 protein is not available, but a structure of bovine P450 21A2 is [42]. More than 80% of the variants known to be adverse from clinical studies can be rationalized in the bovine structure (although more details of exactly why these are debilitating will require more study) [42, 2400].

The published bovine P450 21A2 structure includes the substrate 17α-hydroxyprogesterone [42]; a human P450 21A2 structure with progesterone is also available [2769]. An interesting feature of the published bovine P450 21A2 structure is the presence of two molecules of the substrate (17α-hydroxyprogesterone) [42]. One is in an appropriate position for 21-hydroxylation, but the other is on the "other" side of the substrate near the heme and not in a position for hydroxylation. Spectral binding and reduction experiments are consistent with the occupancy by two substrates, as well as the crystal structure [42]. Dual occupancy does not lead to cooperative interaction [2769].

9.7.47.6 Inhibitors

Relatively little has been published about inhibitors. Detrimental effects of spironolactone have been attributed to inhibition of 21-hydroxylation [2427], although further details with this P450 are lacking. Recently Auchus and his associates [2205] reported that the enantiomeric form of progesterone (*ent*-progesterone) is a competitive inhibitor of P450 21A2 (although not as effective as with P450 17A1). Apparently no new inhibitors of P450 21A2 have been published since the last edition of this chapter was published [149].

9.7.47.7 Clinical Issues

As mentioned earlier, the incidence of defects is relatively frequent and the ability to form cortisol is a problem. Patients who cannot synthesize sufficient aldosterone may lose sodium balance and can develop a fatal "salt-wasting" syndrome. Treatment involves administration of mineralocorticoids and glucocorticoids. Females with severe, classic P450 21A2 deficiency are exposed to excess androgens prenatally and born with virilized external genitalia, but prenatal diagnosis permits prenatal treatment of affected females [2414].

For a review of aspects of P450 21A2 diagnosis and management (in adolescents), see Lin-Su et al. [2428]. In addition to adrenal hyperplasia, P450 21A2 insufficiency has also been considered in relationship to bone density [2429], adrenal mass [2430], Cushing's disease [2431], risk of cardiovascular disease [2432], virilization of female genitalia [2433], and female [2434] and male [2435] infertility. See also [2436, 2437] for more on virilizing congenital adrenal hyperplasia.

Autoantibodies against P450 21A2 have been detected in autoimmune Addison's disease patients [1971].

9.7.48 P450 24A1

The next three P450s (24A1, 27A1, 27B1) are involved in vitamin D metabolism (Fig. 9.29). All three are mitochondrial and receive electrons from the iron–sulfur protein adrenodoxin (via the flavoprotein NADPH-adrenodoxin reductase) (Table 9.2).

9.7.48.1 Sites of Expression

The 24-hydroxylation of 25-hydroxyvitamin D_3 has long been known to occur in the kidney mitochondrial membrane [2438]. Following the purification of a rat P450 with this activity [2439], cDNA clones for chicken [2440] and human [2441] homologs were obtained.

The enzyme is expressed in both proximal and distal kidney tubules [2442] but has also been found in human non-small cell lung carcinomas [2443]. This would appear to be a relatively low abundance P450. Expression has also been reported in human keratinocytes [2444, 2445], colon carcinoma cells [2446], and prostatic cancer cells [2447].

P450 24A1 has also been found expressed in the male reproductive tract [2448], and expression at the annulus of human spermatozoa has been considered as a marker of semen quality [2449].

Peptides corresponding to P450 24A1 have been identified in human liver tissue [635].

9.7.48.2 Regulation

The regulation of the *CYP24A1* gene appears to be complex, although some phenomena observed in animal models have not been examined in as much detail in humans. The activity has long been known to be inducible by vitamin D, perhaps to relieve the cells of an overload, and a VDR element has been found in the 5′-region of the *CYP24A1* gene [2450, 2451]. Parathyroid hormone and cyclic AMP both enhance induction by the VDR [2442].

In human keratinocytes, P450 24A1 mRNA was also elevated by 1α,25-dihydroxyvitamin D_3 [2444]. Studies with rat systems indicate that this response is also mediated by VDR response elements and that two of these (VDRE-1, VDRE-2) operate synergistically [2452]. A functional Ras-

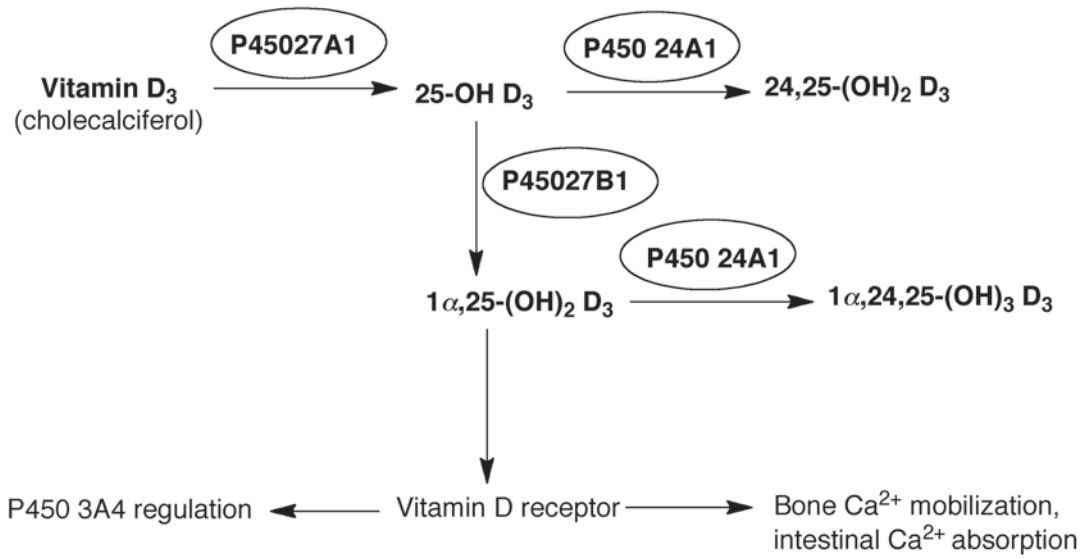

Fig. 9.29 Overview of P450s involved in key steps of vitamin D activation [47]. (With kind permission from Springer Science + Business Media: [149], Fig. 10.16)

dependent Ets-binding site is located downstream from the proximal vitamin D response element (VDRE) site and was critical; the model indicates transcriptional cooperation between Ras-activated Ets proteins and the VDR–RXR complex in mediating 1α,25-dihydroxyvitamin D action on the P450 24A1 promoter [2453]. The YY1 transcription factor has been reported to repress 1α,25-dihydroxyvitamin D_3-induced transcription in cell culture [2454]. The isoflavone genistein was reported to block the transcription of the *CYP24A1* gene in human prostatic cancer cells, and this block could be relieved with the histone deacetylase inhibitor trichostatin A [2447]. Finally, the earlier results with Ets proteins (*vide supra*) have been expanded to show distinct roles of the MAP kinases ERK1/ERK2 and ERK5 [2455]. Induction of P450 24A1 by 1α,25-dihydroxyvitamin D_3 involves Ets-1 phosphorylation at Thr-38, but 1α,25-dihydroxyvitamin D_3 stimulation of ERK1/ERK2 required RXRα phosphorylation on Ser-260 [2455].

1α,25-Dihydroxyvitamin D_3 has been reported to induce P450 24A1 (mRNA) expression in colon cells [2456]. The human P450 24A1 promoter has been characterized, and a short vitamin D stimulating element (VSE) found in rat is absent [2457]. Induction of human P450 24A1 by 1α,25-hydroxyvitamin D_3 is dependent upon a promoter region spanning nucleotides -470 to -392 [2457]. Both a proximal and a downstream element VDR element bind the VDR/RXR heterodimer and somehow lead to induction [2458]. Coregulators are also involved and are responsible for increased RNA polymerase II activity and histone H_4 acetylation.

PXR (liganded) can activate the P450 24A1 gene by directly binding to and transactivating vitamin D-responsive elements within the promoter region [2459].

Vitamin D_3 activates the P450 24A1 promoter by dissociating the corepressor silencing mediator for retinoid and thyroid (SMRT) hormone receptors from the VDR on those VDREs. PXR strongly represses vitamin D_3 activation of the P450 24A1 gene indirectly by binding to and preventing vitamin D_3-dependent dissociation of SMRT from the P450 24A1 promoter. The degree of the PXR-mediated locking of SMRT depends on the relative concentration of vitamin D_3 to the human PXR activator rifampicin; SMRT increases dissociation as this ratio increases. CAR is also found to prevent dissociation of SMRT from the *CYP24A1* promoter [2459]. An SNV in the promoter blocks protein binding and gene activation [2460]. P450 24A1 is also regulated by proinflammatory cytokines (in a cultured human trophoblast system).

P450 24A1 also appears to be subject to epigenetic regulation. Studies in human prostate cancer cells show that repression of the expression of the gene is mediated by promoter DNA methylation and repressive histone modifications [2461]. Placental-specific gene methylation has also been reported [2462]. Along with other evidence for epigenetic control (in prostate cancer cells) [2461], evidence for a change in gene copy number has been reported [2463, 2464]. The DNA methylation levels have been reported to predict vitamin D response variation [1187].

9.7.48.3 Genetic Variation

Genetic variations in P450 24A1 are known and have been considered in the context of clinical issues regarding vitamin D [2465–2468].

9.7.48.4 Substrates and Reactions

Both 25-hydroxyvitamin D_3 and 1α,25-dihydroxyvitamin D_3 are substrates for 24-hydroxylation (Fig. 9.29), with the latter being the preferred substrate [2469]. However, human P450 24A1 can also catalyze other side-chain reactions (Fig. 9.30). [2470, 2471]. Studies with side chain-fluorinated vitamin D analogs also provide evidence for some flexibility of this side chain in allowing P450 24A1 to oxidize different sites [2472, 2473]. Rat P450 24A1 differs from the human ortholog in taking 1α,25-dihydroxyvitamin D_3 on to calcitroic acid instead of the products shown in Fig. 30 [2474–2476].

A number of additional studies with substrate analogs have appeared since the last edition of this book was published [149]. These include the metabolism of A-ring diastereomers of 1α,25-dihydroxyvitamin D_3 [2477]. The 23-hydroxylation occurs, and 25,26,27-trinor-23-ene vi-

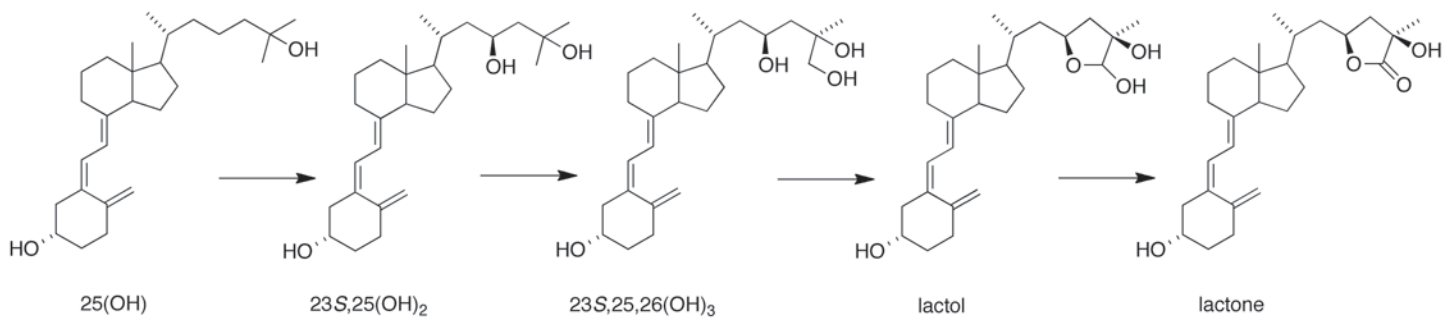

Fig. 9.30 C-23 hydroxylation pathway for 25-hydroxyvitamin D_3 (25(OH)) oxidation catalyzed by P450 24A1 [2470]. (With kind permission from Springer Science + Business Media: [149], Fig. 10.17)

tamin D_3 and 25,26, and 27-trinor-23-ene-1α vitamin D_3 are the respective products formed from 25-hydoxy- and 1α,25-dihydroxyvitamin D_3, both oxidized by P450 24A1 [2478]. Other work showed that different 2α-substituted 1α,25-dihydroxyvitamin D_3 analogs were processed in different ways [2479]. 25-Hydroxy-19-nor- and 1α,25-dihydroxy-19-nor vitamin D_3 are substrates [2480]. Several pathways of oxidation were seen with 1α-propoxyl-1α,25-dihydroxyvitamin D_3 [2481]. Urushino et al. [2482] reported that 1α,25-dihydroxyvitamin D_2 (differing from vitamin D_3 only in the presence of a double bond at the 22, 23 position) is converted into at least ten products by human P450 24A1 (but only to one by rat P450 27A1).

A single (A326G) mutation has been shown to convert human P450 24A1 from a 24- into a 23-hydroxylase [2483]. Also, a V391 L mutation of human P450 24A1 changed the site of hydroxylation to C-25 [2484].

9.7.48.5 Structure

Several reports involved site-directed mutagenesis and homology modeling to gain insight in the structure of P450 24A1 [2485–2487]. However, given the diversity of products observed with minor modifications of either the substrate or protein (Sect. 7.48.4), it is difficult to make definite conclusions from much of this work. An X-ray crystal structure of rat P450 24A1 has been published [43], the first structure of a mitochondrial P450. The structure is an open form, without a substrate, although a 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) detergent molecule is present in the structure. The substrate was docked into the structure.

9.7.48.6 Inhibitors

As discussed with other enzymes involved in vitamin A or D metabolism, there is interest in developing inhibitors of vitamin D degradation as opposed to administration of vitamin D itself, to raise levels of active vitamin D metabolites. Schuster [2445, 2488, 2489] has identified some inhibitors that differ in their selectivity between P450 24A1 and P450 27B1 and have sub-μM IC_{50} values. More inhibitors have been published in the literature [2490–2492]. Some of these are of interest in specifically inhibiting P450 24A1 in cancer therapies related to vitamin D [2493–2495].

9.7.48.7 Clinical Issues

The scheme presented in Fig. 9.29 depicts P450 24A1 as an enzyme involved in deactivating the activated form of vitamin D. The possibility has been considered that defects in P450 24A1 might lead to hypervitaminosis D [47]. An overactive P450 24A1 could lead to vitamin D deficiency. Henry [2496] has reviewed the role of P450 24A1 and made comparisons to other "multistep" P450 enzymes. The possibility is raised that P450 24A1 could serve to generate products with their own biological activities, with P450 24A1 thus being involved in an anabolic pathway. Transgenic rats overexpressing (rat) P450 24A1 were found to have *low* plasma levels of 24,25-dihydroxyvitamin D_3 [2497], which was unexpected. Further, the transgenic rats developed albuminuria and hyperlipidemia shortly after weaning and later developed atherosclerotic lesions in the aorta. These results raise the possibility that P450 24A1 is involved in functions other than vitamin D metabolism [2497].

P450 24A1 can be an issue in situations involving changes in levels of active forms of vitamin D. Some aspects involve cancer; further, P450 24A1 has been considered as a biomarker for some cancers [2448, 2465, 2498–2500]. P450 24A1 has also been considered in the context of kidney disease [2501, 2502]. Because of the relationship of vitamin D with bone, loss-of-function P450 24A1 genetic variants are an issue in hypercalcemia [2503, 2504].

9.7.49 P450 26A1

9.7.49.1 Sites of Expression

P450 26A1 is expressed mainly in liver [2505–2507]. The level is not high, i.e., highest value 2.8 pmol/mg microsomal protein [2508]. Expression of P450 26A1 at the mRNA level is also considerable in brain, lung, and artery, with the highest brain levels being in the cerebral cortex, hippocampus, and temporal lobe [2506]. Ex-

pression is also seen in testis and uterus. At the protein level, the highest expression was in lung, pancreas, and uterus [2506], with some in adipose, intestine, skin, and spleen.

P450 26A1 is present at an earlier stage of embryonic development than P450 26B1 or 26C1 [2509].

9.7.49.2 Regulation

P450 26A1 is regulated by its substrate, retinoic acid, via RAR. Zhang et al. [2510] analyzed a 2.2-kb 5-flanking region of the human *CYP26A1* gene and identified three conserved hexametric direct repeat -5 elements for RAR binding (RARE -1, -2, -3) and an RAR element half-site. The combined element was functional in HepG2 cells. Their results suggest a cooperative model in which the binding to multiple RAR elements may account for the strong inducibility of P450 26A1 in liver, possibly involving looping of the distal region to position it close to the transcription start site [2510].

RAR_{α} is considered to be the major RAR form responsible for the induction of P450 26A1 (and RAR_{β}) in HepG2 cells [2507]. The PPARγ agonists proglitazone and rosiglitazone upregulated P450 26A1 expression tenfold (in HepG2 cells). Further work by Tay et al. [2507] indicated differences in the regulation of P450 26A1 and 26B1. The alteration of P450 26A1 regulation by drugs may have relevance to therapy and drug safety [2507].

A number of other chemicals have been reported to regulate P450 26A1 in rodents and other experimental models [2507], although the relevance to humans has not been established.

9.7.49.3 Genetic Variation

In adult human liver, the levels of P450 26A1 are highly variable [2509], but this has been attributed to the variability of vitamin A intake. At least four alleles have been identified (http://www.cypalleles.ki.se). Two have been linked to lower activity [2511].

9.7.49.4 Substrates and Reactions

Only two substrates of P450 26A1 are known, all-*trans*-retinoic acid and 9-*cis*-retinoic acid. The latter is a much poorer substrate for P450 26A1 (but not for P450 26C1 [2509]). All-*trans*-retinoic acid forms 4(*S*)-hydroxyretinoic acid [667]. Both (*R*) (formed by some other P450s) and (*S*)-4-hydroxyretinoic acid are substrates for P450 26A1, yielding 4-oxo-retinoic acid [667]. Other products are 4,18-dihydroxyretinoic acid and 4-oxo-18-hydroxyretinoic acid (plus 18-hydroxyretinoic acid) [667, 2509].

Although all-*trans*-retinoic acid can be oxidized by P450 3A subfamily enzymes and P450 2C8, P450 26A1 is the predominant enzyme involved in retinoic acid oxidation [2508].

9.7.49.5 Structure

No crystal structures of P450 26A1 are available. A homology model based on P450 3A4 has been published [667].

9.7.49.6 Inhibitors

There is interest in inhibition of endogenous retinoid degradation as an alternative to administration of retinoids [2512]. A number of inhibitors of P450 26A1 are known, the best ones containing imidazoles and triazoles, some having sub-μM IC_{50} values. One novel approach is use of a vaccine targeting (mouse) P450 26A1 as an immunopreventive strategy to prevent breast carcinoma. Selectivity between P450s 26A1 and B1 (and C1) is an issue, although realistically inhibiting both (all three) is a part of the overall strategy [2509, 2512–2520]. Gudas has shown a role of P450 26A1 in stem cell differentiation, and blocking P450 26A1 may be an approach in cell/differentiation therapy to treat neurodegenerative diseases [2521].

9.7.49.7 Clinical Issues

P450 26A1 has considerable relevance in developmental biology, at least in model organisms, because of the control of retinoid homeostasis. *CYP26A1*$^{(-/-)}$ mice show distinct malformations and lethality [2507]. P450 26A1 knockout embryonic stem cells have been reported to exhibit reduced differentiation and growth arrest when retinoic acid is added [2522].

Malfunction of P450 26A1 in retinoid homeostasis is the major clinical issue. One study [2523] suggests a role of P450 26A1 genetic variation in

nonsyndromic bilateral and unilateral optic nerve aplasia. Genetic variation of P450 26A1 has been suggested to be involved in spina bifida [2524] and caudal regression syndrome [2525]. P450 26A1 has been considered in the context of abnormalities of limb development [2526].

9.7.50 P450 26B1

9.7.50.1 Sites of Expression

P450 26B1 was originally identified as a "second" P450 family 26 gene in zebra fish and humans [2527], with a prominent brain localization [2528]. In contrast to P450 26A1, P450 26B1 is not expressed in human liver [2506]. Expression (at the mRNA level) is seen at the highest levels in brain (cerebellum, cerebral cortex, hippocampus, temporal lobe), vein, artery, adipose, bladder, kidney, testis, and skin [2506]. At the protein level, the highest expression was seen in uterus, pancreas, lung, skin, and spleen, with some seen in intestine, adipose, and kidney [2506].

During fetal development, levels in cephalic tissues are tenfold higher at days 57–100 than in later gestation (112–224 days).

9.7.50.2 Regulation

As in P450 26A1, P450 26B1 is also induced by (all-*trans*)-retinoic acid [2505, 2509]. The transcription factors SOX9/SF-1 and FOXL2 have been reported to be antagonistic regulators of P450 26B1 during gonadal development (in mice) [2529]. Expression of P450 26B1 in T cells is inhibited by TGF-β [2530]. As with P450 26A1, PPARγ agonists regulate P450 26B1 transcription and a PPARγ antagonist (the latter effect in contrast to P450 26A1) [2507].

9.7.50.3 Genetic Variation

Genetic variation is recognized in the *CYP26B1* gene. Possible linkages to Crohn's disease [2531] and congenital limb deficiencies [2526] have been proposed.

9.7.50.4 Substrates and Reactions

The substrates and reactions are essentially the same as for P450 26A1; i.e., all-*trans*-retinoic acid is oxidized to the 4- and 18-hydroxy products and the 4-alcohol is further oxidized to 4-oxo derivatives [2509]. 9-*cis*-Retinol and other retinoids are poor substrates.

9.7.50.5 Structure

No crystal structure is available. At least one homology model has been published [2532].

9.7.50.6 Inhibitors

As discussed under P450 26A1 (Sect. 7.49.6, *vide supra*), there is a general concept of giving drugs to block the metabolism of endogenous retinoids rather than administering retinoids themselves [2512]. Most inhibitors of retinoid oxidation have been developed as general inhibitors and do not distinguish between P450s 26A1 and 26B1 [2512, 2515].

9.7.50.7 Clinical Issues

Animal studies show that P450 26B1, like P450 26A1, is important in development [2509]. Some possible outcomes of genetic variations are mentioned in Sect. 7.50.3. P450 26B1 plays a major role in retinoid metabolism and signaling in human aortic smooth muscle cells. The potential for inhibition has already been addressed.

9.7.51 P450 26C1

9.7.51.1 Sites of Expression

In contrast to the other P450 family 26 P450s, P450 26C1 appears to be expressed mainly during embryonic development, at least in animal models [2509]. Sites of expression include hindbrain, inner ear, first bronchial arch, tooth buds, and equatorial retina (of mice) [2533, 2534] However, low levels (of mRNA) can be detected in adult adrenal gland, lung, spleen, testis, and brain [2509, 2535]. Expression is also seen in keratinocyte cell lines treated with 9-*cis*- or all-*trans*-retinoic acid [2509].

9.7.51.2 Regulation

Relatively little information is available, particularly in humans. As indicated above, in keratino-

cyte cultures transcription is induced by retinoic acid [2509].

9.7.51.3 Genetic Variation

Some maladies have been suggested to be linked to genetic variation in *CYP26C1,* including focal facial dermal dysplasia type IV [2536] and nonsyndromic bilateral and unilateral optic nerve aplasia [2523].

9.7.51.4 Substrates and Reactions

P450 26C1 oxidizes both *9-cis*- and all-*trans*-retinoic acid to the 4-hydroxy, 4-oxo-, and 18-hydroxy products (and combinations) [2509, 2537]. These are inactivated (with regard to retinoid receptors) and are the same products formed by P450s 26A1 and 26B1 [2509].

9.7.51.5 Structure

No information about the structure of P450 26C1 is available.

9.7.51.6 Inhibitors

Blocking the metabolism of endogenous retinoids is considered an alternative to administration of retinoids. Some azoles are in development as inhibitors, but it is not clear if their selectivity towards individual 26C family P450s (or others) has been studied and reported yet [2509, 2538].

9.7.51.7 Clinical Issues

The potential clinical issues related to P450 26C1 have not been considered in detail but would involve issues with retinoids, i.e., lack of retinoid metabolism would lead to an overload of retinoid-induced problems. As mentioned above (Sect. 7.51.3), some possibilities exist with genetic variations in focal facial dermal dysplasia type VI and nonsyndromic bilateral and unilateral optic nerve aplasia.

9.7.52 P450 27A1

This is a mitochondrial enzyme that was characterized on the basis of two rather divergent catalytic activities, the 25-hydroxylation of vitamin D_3 (Fig. 9.29) and the oxidation of cholesterol at the C27 position (Fig. 9.31). Thus, the enzyme bridges between hormone (vitamin D) and oxysterol pathways, and the clinical relevance of P450 27A1 is considerable.

9.7.52.1 Sites of Expression

The enzyme is localized in liver mitochondria. Some confusion existed in the early literature because some animal species have liver microsomal vitamin D_3 25-hydroxylases (e.g., hog liver and kidney P450 2D25 [2540, 2541]), but not humans [2542]. The rat and human liver mitochondrial P450 27A1 recombinant enzymes were clearly shown to catalyze both vitamin D_3 25-hydroxylation and the 27-hydroxylation of the side chains of cholesterol and several derivatives [2543, 2544].

Expression, at least at the mRNA level, has also been reported in leukocytes [2545], skin fibroblasts [2546], kidney [2547] (and fetal liver and kidney [2547]), and the arterial wall [2548]. Other sites (in humans) include the male reproductive tract (round and elongated spermatids, vesicles within the caputepididymis, glandular epithelium of canda epididymis, seminal vesicles, prostate, and spermatozoa [2448] and retina pigment epithelial cells [2549, 2550]). P450 27A1 has been detected in human liver using LC–MS [635].

9.7.52.2 Regulation

Several aspects of regulation of the *CYP27A1* gene have been studied. In rats, the enzyme can be induced by gonadotropin [2551]. A hamster model showed downregulation of the gene in cholestatic liver [2539], although human P450 27A1 (used in HepG2 cells) was not subject to negative feedback regulation [1730].

Since the previous edition of this chapter [149], the literature has several additions, indicating that regulation of expression is even more complex. Androgens upregulate expression in HepG2 cells, utilizing a region upstream from −792 [2552]. Estrogens downregulate expression via both ER α and β pathways [2552]. Expression is also regulated by T Fβ1 [2553], RXR, and PPARγ pathways [2554, 2555], and PXR [2556] pathways. There is some discrepancy as to

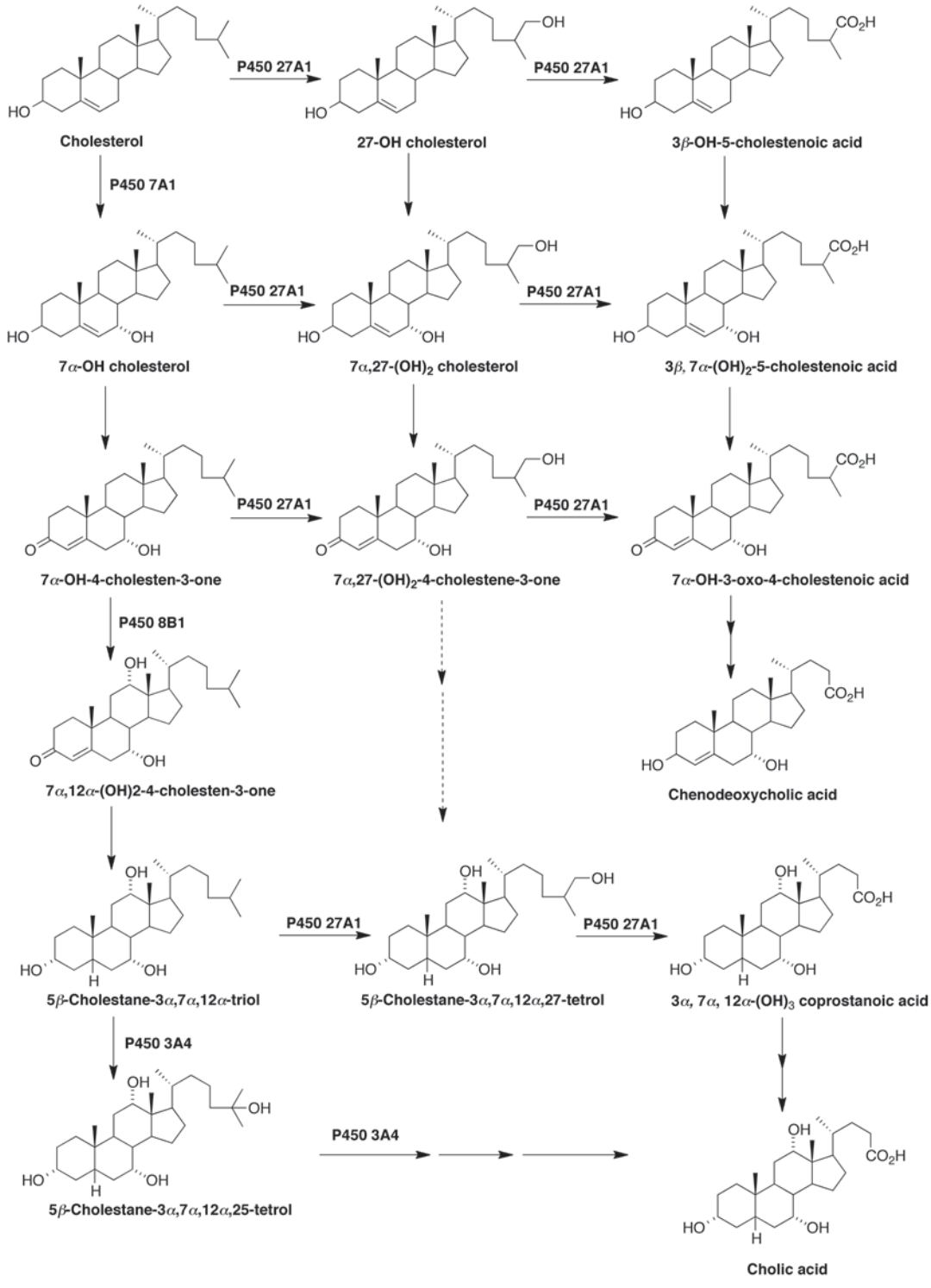

Fig. 9.31 Bile acid synthesis from cholesterol [2539]. The steps shown with *dashed arrows* are tentative. (With kind permission from Springer Science + Business Media: [149], Fig. 10.18)

the involvement of LXR [2554, 2555] in human macrophages. Phenobarbital induces P450 27A1 [2557], although it is unknown if this involves PXR or CAR.

One report involves epigenetic control, i.e., DNA methylation [1187].

9.7.52.3 Genetic Variation

In "normal" human population, the variation in the steady-state P450 27A1 mRNA level was reported to be ~25-fold, compared with 60-fold for P450 7A1 in the same study [1730]. However, at least two polymorphisms (≥1% incidence, no dramatic effect) and 42 other genetic variants (rare alleles, usually debilitating) are known [2546, 2558]. Truncation mutations are known [2545], as well as splice variants [2559]. Defects in the *CYP27A1* gene are associated with a condition known as cerebrotendinous xanthomatosis, a rare, autosomal recessive disorder characterized by accumulation of cholestanol and cholesterol in many tissues. The clinical manifestations include tendon xanthoma, premature cataracts, juvenile atherosclerosis, and a progressive neurological syndrome involving mental retardation, cerebellar atoxia, pyramidal tract signs, myelopathy, and peripheral neuropathy [47, 2558].

The known variants leading to cerebrotendinous xanthomatosis have been reviewed [2560]. Mutation in the gene has been associated with fatal cholestasis in infancy [2561]. Variants have also been linked to susceptibility to amyotrophic lateral sclerosis [2562].

9.7.52.4 Substrates and Reactions

Expanding on the previous discussion, P450 27A1 catalyzes the 25-hydroxylation of vitamin D_3 (Fig. 9.29), 1α-hydroxyvitamin D_3, vitamin D_2, and 1α-hydroxyvitamin D_2 and also the 27-hydroxylation of cholesterol and several derivatives (Fig. 9.31) [2563, 2564]. The cholesterol alcohols are further oxidized by the enzyme to aldehydes and then carboxylic acids [2565]. The available information suggests release of the intermediates in the pathway [2565]. The regioselectivity of the enzyme is considered to be a function of the distance of the hydroxylation site to the end of the side chain [2566].

More detailed analysis of the vitamin D_3 reaction has been done with *E. coli* recombinant P450 27A1, with evidence for the following products (from vitamin D_3): 25-hydroxy, 26-hydroxy, 27-hydroxy, 24*R*,25-dihydroxy, 1α,25-dihydroxy, 25,26-dihydroxy, 25-,27-dihydroxy, 27-oxo, and an unidentified dehydrogenated product [2544, 2567].

P450 27A1 occupies a place at the intersection of metabolism of vitamin D (a secosteroid) and sterol metabolism (Table 9.1), and perhaps because of this less than stringent substrate selectivity, the list of substrates and reactions continues to grow. 2α-Propoxy- and 2α(3-hydroxypropoxy)-1α,25-dihydroxyvitamin D_3 are substrates. Human P450 27A1 also converted 25-hydroxyvitamin D_3 into 1α,25-dihydroxyvitamin D_3 and 25,27-dihydroxyvitamin D_3, as well as the conversion of 25-hydroxyvitamin D_3 into 4β,25-dihydroxyvitamin D_3 [2568]. Thus, 4β-hydroxylation was catalyzed.

In the retina, P450 27A1 catalyzed the conversion of the oxysterol 7-ketocholesterol to 27-hydroxy and 27-carboxy products [2549]. Further work by Pikuleva and her associates showed that several other cholesterol precursors (in addition to Δ^7-dehydrocholesterol) are substrates (for 27-hydroxylation), including desmosterol, zymosterol, and lanosterol [2569]. The dehydrocholesterol products (25-hydroxy and 26/27-hydroxy) modulate LXR activity [2570]. Progesterone is a substrate, undergoing reduction of the C-20 keto group (to an alcohol) [2571].

A study of the selectivity of sterol analogs as substrates indicates that more polar derivatives (e.g., cholesterol sulfate) are better substrates [2572]. Sterols with a 3-oxo-Δ^4 structure were found to be hydroxylated at higher rates than 3-hydroxy-Δ^5 analogs. The very high activity with the cholestanol precursor 4-cholesten-3-one may be of importance in the accumulation of cholestanol in patients with cerebrotendinous xanthomatosis disease.

9.7.52.5 Structure

Some information about the roles of amino acids can be inferred from the knowledge of alleles involved in cerebrotendinous xanthomatosis; many

of these proteins were unstable when attempts were made at heterologous expression [2573]. Other work by Pikuleva et al. [2574] with the putative F and G helices has shown differences due to substitution of Phe-207, Ile-211, Phe-215, Trp-235, and Tyr-238. Interestingly, the I211K and F215K mutations affected the regioselectivity and enabled the enzyme to catalyze C–C bond cleavage. Further work with mutants in this region led to weaker association of P450 27A1 with the membrane, and some of the nonconservative changes yielded impaired catalytic activity [2575].

Human P450 27A1 can be contrasted with porcine P450 2D25, which also catalyzes vitamin D_3 25-hydroxylation. The only human subfamily 2D P450 enzyme is P450 2D6, which does not have activity towards vitamin D. Further, changing a set of residues of porcine P450 2D25 to their counterparts in (human) P450 2D6 abolished the activity towards vitamin D_3 [2576].

No structures have yet appeared but more models have, some based on site-directed mutagenesis work [2483, 2577, 2578].

9.7.52.6 Inhibitors

Apparently, little specific work has been done on inhibition of this enzyme. Inhibition of this enzyme by a drug would probably be undesirable.

9.7.52.7 Clinical Issues

Low serum 25-hydroxyvitamin D_3 concentrations have been reported in a variety of other medical conditions and are considered to be a potential problem [2579]. Although cerebrotendinous xanthomatosis is linked with defective P450 27A1 [47], there are a number of enigmas about the etiology. A heterozygote showed frontal lobe dementia and abnormal cholesterol metabolism [2580]. Compound heterozygous mutations have also been reported to cause a variation of cerebrotendinous xanthomatosis [2558]. P450 7A1 may also play a role in the etiology of the disease [1778].

Björkhem [2581] has recently reviewed the issue of whether oxysterols (e.g., hydroxycholesterol) control cholesterol homeostasis. Studies with rodents and cultured cells have not been very clear to date. For instance, disruption of the mouse *Cyp21a1* gene yielded reduced bile acid synthesis but no change in levels of cholesterol or 1α,25-dihydroxyvitamin D_3 [2582]. P450 27A1 is constitutively expressed in the normal artery wall and is substantially upregulated in atherosclerosis, and the possibility has been raised that P450 27A1 may be a protective mechanism for removing cholesterol [2548]. Further, immune complexes and IFN-γ decreased P450 27A1 expression in human aortic endothelial cells, peripheral blood mononuclear cells, monocytes-derived macrophages, and a human monocytoid cell line, suggesting downregulation of P450 27A1 to maintain cholesterol homeostasis in the arterial wall [2583].

In *Cyp27a1-/-* mice, a dramatic increase in the level of P450 3A enzymes was seen; some sterols accumulate and induce via the mouse PXR system [2584]. P450 3A4 has some side-chain hydroxylation activities towards cholesterol-derived sterols [1328]. However, elevated P450 3A4 activity was not increased in cerebrotendinous xanthomatosis [1328], indicating a difference in the murine and human systems. Recently Escher et al. [2585] have reported that cholesterol efflux in CHOP cells is enhanced by heterologous expression of human P450 27A1, and the authors suggest this as part of a protective system against atherosclerosis. The basis is probably the ability of 27-hydroxycholesterol to act as an endogenous ligand for the LXR in cholesterol-loaded cells [2586].

In considering the general question of whether oxysterols (e.g., 27-hydroxycholesterol) control cholesterol homeostasis, the hypothesis is still open and the rodent data are not totally clear here. Björkhem [2581] has made the point that humans lacking P450 27A1 have normal circulating levels of cholesterol.

Reference has already been made to genetic variants in Sect. 7.52.3 (*vide supra*). A genetic association with obesity traits has been considered [2587], as well as with coronary artery disease [2588]. The area of cerebrotendinous xanthomatosis has been reviewed recently, including P450 27A1 [2589].

The production of 27-hydroxycholesterol by (P450 27A1) has been linked to breast cancer pathophysiology, in that it serves as an ER and

LXR ligand and increases ER-dependent growth and LXR-dependent metastasis in a mouse models of breast cancer [2590]. Accordingly, lowering circulating cholesterol levels and inhibiting P450 27A1 have been proposed as mechanisms to treat breast cancer.

9.7.53 P450 27B1

As discussed earlier, vitamin D is an important hormone. A critical step in activation is 1α-hydroxylation [2591] (Fig. 9.29). Early work established the P450 nature of the enzyme, localized in kidney mitochondria [2592]. Subsequent work demonstrated that the 1α- and 24-hydroxylation activities could be attributed to different enzymes [2593, 2594]. Some early work had suggested that the 1α- and 25-hydroxylation activities were associated with the same enzyme [2595], but later work showed that these activities were due to P450 27B1 and 27A1, respectively.

9.7.53.1 Sites of Expression

The cloning of the human cDNA for what is now known as P450 27B1 established kidney mitochondrial P450 27B1 as the vitamin D_3 1α-hydroxylase [2596].

P450 27B1 is expressed in many parts of the human kidney, including the distal convoluted tubule, the cortical and medullary part of the collecting ducts, and the papillary epithelia [2597]. Lower expression was observed along the thick ascending limb of the loop of Henle and Bowman's capsule. Some, weaker expression was observed in glomeruli or vascular structures. In normal humans, the distal nephron is the predominant site of expression [2597].

P450 27B1 is also expressed in many extrarenal sites (human) where it is involved in vitamin D-related activities, including skin (basal keratinocytes, hair follicles), lymph nodes (granulomata), colon (epithelial cells and parasympathetic ganglia), pancreas (islets), adrenal medulla, brain (cerebellum and cerebral cortex) [2598], placenta (decidual and trophoblastic cells) [2598–2600], cervix [2601], and parathyroid glands [2602]. Thus, P450 27B1 may be an intracrine modulator of vitamin D function in peripheral tissues [2598]. The expression of P450 27B1 was elevated in parathyroid adenomas but attenuated in carcinomas, relative to normal parathyroid tissue [2602]. P450 27B1 has also been found in (human) and endometrial tissue [2603]. For reviews on the significance of extrarenal P450 27B1, see [2604, 2605].

9.7.53.2 Regulation

Although the *CYP27B1* gene is only 5 kb in size [2606], the regulation is quite complex. The promoter is in the −85/+22 region and requires a functional CCATT element. Three consensus AP-1 sites are upstream [2607]. Enzyme activity has long been known to be stimulated by low phosphorus diets (in animal models) [2608], and more recently this phenomenon has been linked to a growth hormone mechanism [2609, 2610]; its relevance in humans is not known.

Complexity is seen in different models. Parathyroid hormone-related protein and Ca^{2+} have conflicting actions in a rude rat model of humoral hypercalcemia of malignancy [2611]. In differentiated Caco cells, there is upregulation of P450 27B1 expression by 1α,25-dihydroxyvitamin D_3 and EGF but downregulation in less differentiated Caco cell lines [2446]. P450 27B1 is regulated by proinflammatory cytokines in human trophoblasts [2612]. Immune regulation of P450 27B1 has been reported in monocytes [2613], and uremia downregulated P450 27B1 in monocytes [2614]. Gene amplification (and splice variants) has been reported in gliobastoma cells [2615]. A number of growth factors have been reported to regulate (mostly suppress) P450 27B1 expression, including growth factor independent-1 (GFI-1) [2616], TGFβ [2617], nuclear receptor 4A2 and CIEBP$_\beta$ [2618], thyroid hormone [2619], and NFκB [2620].

Regulation of P450 27B1 expression by (the product) 1α,25-dihydroxyvitamin D_3 has been reviewed [2621]. The product downregulates P450 27B1 in colon cells [2456, 2622]. Part of the mechanism has been attributed to hypermethylation [2623], although increased copy number (and not hypomethylation) has been identified as the cause of overexpression in colorectal cancer [2464].

9.7.53.3 Genetic Variation

Another aspect of regulation of P450 27B1 is genetic; P450 27B1 deficiency results in type I vitamin D-dependent rickets [2624]. The genetics have been established in more than 20 variants [2625, 2626]. At least 13 missense variants have been observed, none of which encode an active protein. Some of the mutants are splicing defects [2627]. Some variants in *CYP27B1* are also involved in what is termed pseudovitamin D-deficiency rickets [2628, 2629].

Since the last edition [149], the genetic information has greatly expanded. The number of variants has increased, and *CYP27B1* associations have been considered with diabetes [2630–2632], brain and skin cancers [2633], Graves' disease, Addison's disease, and Hashimoto's thyroiditis [2631, 2634–2636], congestive heart failure [2637], and multiple sclerosis [2638–2640].

The biochemical effects of the variants are reviewed in [2623, 2641]. Perhaps the most biologically plausible relationships of P450 27B1 variants are with rickets disease type I [2642] and fracture risk in the elderly [2643].

9.7.53.4 Substrates and Reactions

P450 27B1 can catalyze the 1α-hydroxylation of both 25-hydroxy and 24(*R*),25-dihydroxyvitamin D_3 [2475, 2644] (Fig. 33). The intrinsic activity (catalytic efficiency, k_{cat}/K_m) for the recombinant human enzyme is better for 24(*R*),25-hydroxy vitamin D_3, but this does not mean that this is the favored substrate in the cell because of the balance of vitamin D metabolites regulated by P450s 24A1 and 27A1 [47]. Apparently, the 25-hydroxy group is an obligatory requirement [2470, 2644].

In addition, 19-nor vitamin D_3 analogs are substrates [2480]. The products of the reactions of P450 11A1 on vitamin D_3 are also substrates [2645, 2646].

9.7.53.5 Structure

No crystal structures have been published.

Some information is available from the natural mutants of P450 27B1, even if the basis for loss of activity is not obvious. Inouye's group [2573] has provided evidence that Arg-107, Gly-125, and Pro-497 are not simply involved in binding substrate but required for proper folding. It was also suggested that Arg-389 and Arg-453 are involved in heme binding and that Asp-164 stabilizes the bundle of the D, E, I, and J helices. Thr-321 is suggested to be involved in O_2 activation [2573]. The natural mutants L343F and E189G show partial activity and the individuals bearing these have only marginal impairment [2647].

Several homology models have been proposed [2476, 2648, 2649].

9.7.53.6 Inhibitors

Little has been done because impairment of this enzyme is a clinical problem. Some thiavitamin D analogs have been evaluated in animal models [2650].

9.7.53.7 Clinical Issues

The significance of the enzyme is due to the pleiotropic actions of the active form of vitamin D, 1α,25-dihydroxyvitamin D_3, which include regulation of calcium homeostasis, control of bone cell differentiation, and modification of immune responses [2651]. The 1α-hydroxylation reaction is rate limiting and hormonally controlled. The expression of the gene is usually tightly regulated (*vide supra*), but gene defects are responsible for type I vitamin D-dependent rickets [2652]. At least 30 different variants are known in patients [2624, 2653]. Even the "mild" phenotype of type I rickets is due to deficiency in P450 27B1 [2654].

Cyp27b1-knockout mice have been characterized and show a typical rickets phenotype [2655]. Another knockout mouse model showed skeletal, reproductive, and immune dysfunction [2656]. Rickets was also observed in a conditional knockout model [2657].

Patients with severe renal insufficiency show attenuated 1α-hydroxylation activity [2658].

Another aspect of P450 27B1 research involves cancer. Increased activity was reported in parathyroid tumors [2659]. Some splice variants of the *CYP27B1* gene (coding for truncated proteins) were amplified in human (brain) gliomas [2660]. Reports have also appeared on the relationship of P450 27B1 expression to various bio-

logical processes in human non-small cell lung carcinomas [2443], colon tumors [2661–2663], and prostate cancers [2664, 2665], generally with decreased expression in tumors.

Finally, 1α,25-dihydroxyvitamin D_3 is used to treat psoriasis, and patients can develop resistance. An experimental model for therapy involves enhancement of the endogenous production of 1α 25-dihydroxyvitamin D_3 by gene therapy [2666].

The potential disease relevance of several genetic variations has already been presented in Sect. 7.53.3 (*vide supra*). Since the previous edition of this chapter [149], vitamin D 25-hydroxylase deficiency has been reviewed [2667, 2668]. Studies with *CYP27B1*-knockout mice have also been reviewed [2669]. Rickets (type I) still appears to be the most relevant issue [2649].

9.7.54 P450 27C1

9.7.54.1 Sites of Expression

P450 27C1 is expressed, at least at the mRNA level, in liver and a number of other tissues, including kidney, pancreas, lung, adrenal, salivary gland, and more [2670]. It is of interest to note that rats and mice do not have this gene.

The sequence identity to P450s 27A1 and 27B1 indicate that it should be a mitochondrial P450, although direct evidence is not available.

9.7.54.2 Regulation

No information is available about the regulation of P450 27C1.

9.7.54.3 Genetic Variation

No information has been published.

9.7.54.4 Substrates and Reactions

The protein was expressed in *E. coli* using an *E. coli*-optimized cDNA [2670]. The purified enzyme, reconstituted with recombinant adrenodoxin and NADPH-adrenodoxin reductase, did not catalyze the oxidation of cholesterol, vitamin D_3, 1α-hydroxyvitamin D_3, or 25-hydroxyvitamin D_3. In other studies, none of a test set of procarcinogens [350] was activated to a genotoxic product.

9.7.54.5 Structures

No structural information is available.

9.7.54.6 Inhibitors

No inhibition results have been published.

9.7.54.7 Clinical Issues

P450 27C1 was a high-frequency gene in an analysis of factors involved in avascular necrosis of the femoral head [2671].

9.7.55 P450 39A1

9.7.55.1 Sites of Expression

Much of our knowledge of this enzyme is still based on animal models. Russell and his associates used expression cloning to characterize a cDNA coding for a 24-hydroxycholesterol 7α-hydroxylase [2672]. Expression in the liver appears constitutive and abundant. Expression has also been detected in the ciliary nonpigmented epithelium of (bovine) eye [2673]. Those studies have not really been extended to humans [2674].

9.7.55.2 Regulation

Very little information is available. One study showed that carbamazepine, a barbiturate-like inducer (using PXR and CAR), upregulated hepatic P450 39A1 mRNA in patients [2675].

9.7.55.3 Genetic Variation

At least three variants have been reported (*rs7761731* (N324K), *rs93981468,* and *rs953062*) [2676, 2677].

9.7.55.4 Substrates and Reactions

All of our knowledge is still based on the presumed similarity to the mouse enzymes [2672]. That expressed enzyme oxidizes (7α-hydroxylation) 24*S*-hydroxycholesterol much more efficiently than 25- or 27-hydroxycholesterol. These results suggest that the enzyme is highly selective for 24*S*-hydoxycholesterol (a product of P450 39A1).

9.7.55.5 Structure

No structures or homology models have been reported.

9.7.55.6 Inhibitors

No inhibitors have been reported.

9.7.55.7 Clinical Issues

Interestingly, at least two reports associate *CYP39A1* SNPs with changes in drug metabolism [2676, 2677]. However, in neither case was the enzyme actually shown to be involved in the metabolism, and one report [2676] has a caveat about a possible artifact with a SNP for a transporter. It is possible that P450 39A1, like P450 46A1 (*vide infra*), may oxidize drugs, but presently there is no other evidence for this.

9.7.56 P450 46A1

9.7.56.1 Sites of Expression

P450 46A1 is characterized as a brain P450. It was identified first (in mice) in a search for extrahepatic enzymes catalyzing the 24-hydroxylation of cholesterol [2678, 2679]. P450 46A1 is also expressed in neurons of the neural retina [2680]. In humans, there is a lack of enzyme and product (24-hydroxycholeseterol) in retina but not in brain [2681]. A heavy isotope (full protein) method was utilized in quantitating P450 46A1 in human brain (temporal lobe) and retina (0.1–0.4 pmol/mg tissue protein) [2682]. None was detected in retinal pigment epithelium.

9.7.56.2 Regulation

One interesting aspect of P450 46A1 is the reported learning disability in *CYP46A1*-knockout mice [2679]. Abnormal induction was reported in glial cells of Alzheimer's disease [2683]. Transcriptional regulation in brain involves Sp factors (Sp3, Sp4) [2684]. Part of this process may involve histone deacetylation [2685]. Neuronal differentiation alters the ratio of Sp transcription factors required for the P450 46A1 promoter. Chromatin-modifying agents increase transcription of P450 46A1, i.e., the demethylating agent 5′-aza-2′-deoxycytidine induced P450 46A1, acting synergistically with trichostatin A in activating transcription. Further work showed that this reagent (azadeoxycytidine) induced gene expression in a DNA methylation-independent mechanism, decreasing Sp3/histone deacetylase binding to the proximal promoter [2686]. Okadaic acid has been reported to inhibit the trichostatin A-mediated expression of P450 46A1 in an ERK1/2-Sp3-dependent pathway [2687].

9.7.56.3 Genetic Variation

Variations in the *CYP46A1* gene have been of interest in large part due to the possible relevance to Alzheimer's disease (*vide supra*) [2388, 2683, 2688–2712]. However, not all studies agree that a relationship exists [2713–2716]. A meta-analysis has been published [2717]. Genetic polymorphisms have been associated with age-related macular degeneration [2718].

9.7.56.4 Substrates and Reactions

Cholesterol 24-hydroxylation is the main reaction ascribed to P450 46A1 [2679]. However, several other sterols are also substrates, including 24(*S*)-hydroxycholesterol (25- and 27-hydroxylations), 7α-hydroxycholesterol, cholestanol, progesterone, and testosterone [2719]. Some drugs are also substrates [2719]. 7-Dehydrocholesterol has been reported not to be a substrate [2720], but if not, then the source of 24-hydroxy-7-dehydrocholesterol is unclear. Recently we have found that recombinant human P450 46A1 catalyzes the 24- and 25-hydroxylation of 7-dehydrocholesterol and the 24*S*,25-epoxidation and 27-hydroxylation of desmosterol, with efficiencies similar to cholesterol [2721].

Interestingly, P450 46A1 binds and oxidizes a number of drugs [2719, 2722]. This is an interesting phenomenon in that most of the P450s that appear to be specialized for oxidation of endogenous substrates do not use xenobiotics as substrates. The overall in vivo contribution of P450 46A1 to the metabolism of these drugs, even in brain, is unknown. Further, P450 46A1 activity (towards cholesterol) is stimulated by binding to some drugs (e.g., efavirenz, acetaminophen, mirtazapine, galantamine), and the in vivo relevance of this effect has been shown in a mouse model [2723].

9.7.56.5 Structure

X-ray crystal structures of P450 46A1 have been reported in the absence and presence of the substrate cholesterol 3-sulfate [2722, 2724]. As with

many other P450s, there is a major conformational change upon binding.

Structures have also been reported with drugs bound [2722, 2725, 2726]. Some of these are substrates.

9.7.56.6 Inhibitors

Largely due to the studies that involved drug binding to P450 46A1, a number of inhibitors have been identified, including fluvoxamine [1962, 2725, 2727]. In a mouse model, in vivo inhibition was reported [2727].

With P450 46A1, there appears to be no impetus to develop an inhibitor, in light of the issues. What is a more important issue is avoiding inhibition, given the literature on the drugs that do this. Several azoles used in the clinic (e.g., posaconazole, voriconazole, clotrimazole) are tightly bound [2726].

9.7.56.7 Clinical Issues

The major clinical issues are the possible genetic links to Alzheimer's disease (*vide supra*) [2712, 2728–2730] and glaucoma [2708]. Other issues are related to brain injury [2731, 2732]. P450 46A1 has also been considered in relation to disease manifestation of acute autoimmune encephalomyelitis (actually the level of 24-hydroxycholesterol) [2733].

9.7.57 P450 51A1

Lanosterol is an important intermediate in cholesterol synthesis, and 14α-demethylation is established as a step in the pathway. Yoshida's laboratory had studied the yeast enzyme for many years and then demonstrated the reaction in rat liver microsomes in 1994 [2734]. Subsequently the reaction was also demonstrated in rat brain microsomes [2735].

The literature associated with P450 51A1 is largely devoted to the enzyme in parasites and to developing approaches to inhibition to treat diseases. The information regarding the human enzymes is more limited, although now there is significant regulatory and structural information.

9.7.57.1 Sites of Expression

Waterman and Rozman identified the human *CYP51A1* gene and two pseudogenes [2736]. mRNA blot analysis showed the highest levels in testis, ovary, adrenal, prostate, liver, kidney, and lung. In mouse testis, P450 51A1 was localized in both round and elongated spermatids [2737]. The enzyme is also found in (rodent) oocytes [2738].

9.7.57.2 Regulation

With regard to regulation of the human gene, primer extension studies indicated predominant transcription initiation sites in liver, lung, and kidney, and placenta 250- and 249-bp upstream from the translation start site and a second major site at −100 bp, with the absence of TATA and CAAT patterns and a GC-rich sequence in the promoter region [2736]. Multiple (rat) testis-specific transcripts arise from differential polyadenylation site usage [2739].

In human adrenocortical H295R cells (in culture), cholesterol deprivation led to a 2.6–3.8-fold induction of P450 51A1 mRNA, which was suppressed by the addition of 25-hydroxycholesterol [2740]. In the liver and other somatic tissues, the P450 51A1 gene is regulated by a sterol/SREBP-dependent pathway [2741]. In testis, cAMP/cAMP-responsive element modulator $CREM_1$-dependent regulation predominates. Sp1 functions to maximize the sterol regulatory pathway of P450 51 [2742].

Insulin is an essential factor in "basal" expression of P450 51A1 in rat liver, with possible involvement of SREBP-1c [2743]. In a porcine vascular endothelial cell model (and in arterial wall), LDLs downregulated P450 51A1 through an SREBP-2 mechanism [2744].

P450 51A1 has been identified as an early response gene [2745]. Hughes et al. [1301] reported that Dap1/PGRMC1 binds and regulates mammalian P450 51A1 (and 3A4), based on work in a yeast model. This result was confirmed for P450 51A1 in mammalian cell culture (HEK 293 and HepG2 cells) [1302].

9.7.57.3 Genetic Variation

The enzyme is also found in (rodent) oocytes [2738].

Rozman and her associates have analyzed genetic variations and reported that the P450 51A1 gene contains fewer variants than any other human P450 gene [2746, 2747]. This may be related to the importance of this enzyme; i.e., abnormalities might be lethal.

9.7.57.4 Substrates and Reactions

Stimulation of human P450 51A1 activity by cytochrome b_5 in a reconstituted system has been reported by Kelly's laboratory [2748].

The normal mammalian substrate for P450 51A1 is lanosterol [2749], with the 14α-demethylation process proceeding in what are assumed to be three consecutive steps, as with some other P450s, e.g., 11A1, 17A1, 19A1 (Fig. 9.22). Interestingly, both human and yeast (*Candida albicans*) P450 51A1 showed relatively little selectivity among a closely related group of analogs [2749]. It is also interesting to note that even though this P450 has a relativity defined role in a physiological process, the kinetic parameters are relatively poor among P450s (k_{cat}/K_m 300 M^{-1}/s) [2749].

9.7.57.5 Structure

Three X-ray crystal structures of human P450 have been reported, ligand-free and with the antifungal drugs ketoconazole and econazole [2750]. As observed with many other P450s, a substantial conformational change in the enzyme occurs on binding ligand. In this case, the changes are in B-helix and F–G loop regions. Azole binding occurs mainly through hydrophobic residues in the active site. Presumably, similar changes would occur upon binding of the substrate lanosterol.

9.7.57.6 Inhibitors

Most of the interest in inhibition has been with fungal P450 51, as a target for antimycotic drugs. The goal is to select candidate drugs inhibitory to fungal P450 51 but not human P450 51.

Some work on the interaction of azoles with human P450 51A1 has been published [2751]. Although human P450 51A1 has been suggested as a target for cholesterol-lowering drugs, apparently little has been done and potential toxicity due to the steroidogenic and potential germ cell side effects (*vide supra*) could be an issue.

A comparison of inhibitors of human and *Candida albicans* P450 51A1 with a series of azoles has been reported [2752], and some more inhibitors have been considered regarding blocking cholesterol synthesis [2753].

9.7.57.7 Clinical Issues

Most of the work discussed here is from experimental studies on the possible role of P450 51A1 in reproduction, and the translation of phenomena from animal models to humans is still somewhat speculatory. However, the very high level of P450 51A1 expression in postmeiotic haploid spermatids is striking. The action of P450 51A1 is proposed to lead to the production of signaling steroids in haploid germ cells [2754]. Meiosis-activating substances (MAS) are produced by 14-reduction of the products of the action of P450 51A1 on lanosterol [2754]. Follicular fluid MAS (FF-MAS) is formed from lanosterol in rat spermatids [2755]. Yoshida's group has reported gonadotropin-dependent expression of P450 51A1 in rat ovaries and the product of MAS [2756].

The reaction and possible physiological significance of the system in reproduction have been reviewed recently by Rozman [2757]. Leidig cells and acrosomes of spermatids have the highest P450 51A1 levels, and primary mouse oocytes and granulose cells also contain P450 51A1. MAS may have a role in fertilization [2757].

As mentioned earlier, P450 51A1 deficiency has been considered in the context of Antley–Bixler syndrome [2758], and *CYP51A1*-knockout mice show a resemblance to this syndrome [2759].

9.8 Conclusions and Future Issues with Human P450

Having just celebrated the 50th anniversary of the discovery of P450 [2760], one can look back on the success in the area of P450s and medicine. The attack on the human P450s did not start in earnest until 20 years after the original discovery of P450 but has proven to be a remarkable success story in the translation of discoveries in basic science. Today we are generally capable of understanding most aspects of (oxidative) drug

metabolism and even predicting it based on in vitro experiments. Medicinal chemists have logical paths to improve human pharmacokinetics. Steroid metabolism can largely be understood at a genetic level, even if the basis for loss of function in each case is not yet known.

Pharmacists can avoid many adverse drug–drug interactions based on knowledge—or electronic histories—of P450 selectivities.

Having accomplished all of this, what does the future hold for P450 research? Clearly, there are still many basic questions, many of which are general questions about P450 function. For instance, the role of cytochrome b_5 in the P450 17A1 17,20-lyase reaction is not solved. More than half of the human P450s do not have crystal structures, and some of these will be problematic until more substrates are discovered. Following are four of the more translational areas in which P450 research can be applied and is needed.

9.8.1 Orphan P450 and Their Reactions

As pointed out in Table 9.1 and the description of individual P450s, relatively little information is still available about this group of the human P450s. Some substrates are being found, both endogenous and xenobiotic, but some of these P450s still have no real substrate at all (e.g., 20A1). There is no compelling evidence that these P450s make major contributions to the metabolism of many drugs (with some occasional exceptions [1214]). Some interesting reactions with endogenous compounds have been identified, but exactly what their physiological importance is remains unknown. The approaches in this area are difficult, but the annotation of functions of genes is probably one of the most important aspects of biochemistry and biology.

9.8.2 Pharmacogenetics and Pharmacogenomics

Advances in recombinant DNA technology, including the completion of the Human Genome Project, have made it possible to rapidly map and screen for SNVs in individuals. Knowledge of P450 SNVs was seen as a major aspect of "personalized medicine" [2761]. However, the development of this area has been somewhat slow since 2000, when "personalized medicine" was touted as "just around the corner." At this time, there is still no drug on the market for which the US Food and Drug Administration or similar agencies in other countries require genetic tests [782]. More recently, some hospitals and medical centers are doing some SNV analysis for a few drugs. As an example, at the author's own institution (Vanderbilt), *CYP2C19* analysis is used with administration of clopidogrel, in order to predict which patients will respond [782, 848–854]. More tests like this will probably follow in the future. (Of course, genotype analysis is already extensively used in drug discovery and development, mainly to avoid drug candidates that might show highly variable pharmacokinetics.)

The major issues in the implementation are added costs (although one can argue that SNV analysis is a cost-effective investment and could reduce hospital stays) and the limited number of proven successes. The author's own opinion is that there will be more progress, particularly with drugs used in oncology, but exactly how fast the field develops is still a matter of speculation.

9.8.3 P450s and Cancer Susceptibility

As mentioned previously, carcinogen metabolism has been one of the drivers for the P450 field. There is strong evidence that the P450 composition can strongly influence chemical carcinogenesis in experimental animal models [2762], reinforced with transgenic mouse models [1441].

Nevertheless, there is still relatively limited evidence that variations in P450 influence cancer risk in humans. As mentioned earlier (Sect. 7.2.7), high P450 1A2 levels can influence colon cancer risk, but only when coupled with an *N*-acetyltransferase polymorphism and high intake of well-done meat [132]. Although a number of P450–cancer relationships have been proposed [108, 109, 123, 1079–1087, 2763, 2764], the evidence is still limited. The difficulty in establishing relationships results from the lack of defined

exposure data available in most cases and the long time period needed to develop cancer. At the molecular level, many P450s not only activate carcinogens but also detoxicate them as well, e.g., aflatoxin B_1 [1331] (Figs. 9.9 and 9.10).

One issue is that epidemiology studies are often initiated in the absence of any information regarding relevant substrates and reactions, and (not surprisingly) weak associations are found and are difficult to repeat. Clearly, more innovative approaches are needed to address the issues.

9.8.4 P450 and Chronic Diseases

In addition to cancer (and endocrine and drug interactions), there is evidence that human P450s can influence chronic diseases (Table 9.3). Several P450s, including those in the 2C, 2J, 4A, and 4F subfamilies, have been proposed to be involved in hypertension, as judged by both basic models and epidemiological studies [2765]. Epidemiological studies may suggest a role of P450 2D6 in Parkinson's disease [953], although the relationship probably has limited support.

Although a degree of skepticism is necessary when considering translational reports regarding P450, what we know about the P450s does argue that we still have important relationships to discover. Several of products of the sterol-metabolizing enzymes are generally powerful biological mediators (e.g., oxysterols that are ligands of nuclear receptors [1715, 1716, 1749]). As in the case of cancer, the differences in life span etc. may be subtle and difficult to detect. Again, new strategies are needed in this area.

My discussion of research needs in the P450 field has been restricted here to the translational aspects of human P450 research. For more consideration of the present state and future direction of P450 research in general, see [2760].

9.9 Some Final Thoughts

Reviewing the progress in research on human P450s in the past 10 years is exciting but also humbling, in that even what I consider to be a productive laboratory of my own contributed < 1 % of that literature. After dealing with all 57 human P450 genes (yielding about the same number of proteins, i.e., see P450s 2A7, 4F3), a few major "take-home" messages can be summarized:

We still have more to learn about some chemical mechanisms (e.g., C-C cleavage), even some that have been thought to be firmly established.

The number of X-ray crystal structures of human P450s is rather amazing (Table 9.1). Nothing seems impossible for the next 10 years. There is a problem in that some P450s do not have substrates yet, and the usefulness of an unliganded P450 structure is limited.

The regulation of many of the P450 genes is quite complex. Finding one regulatory factor leads to another.

The "orphan" P450s (those without established function) (Table 9.1, see also [149]) are falling into categories, at least in terms of some of the reactions that they can do. Some of those were previously in the "orphans" category [149] now have as much information as some already in the xenobiotics or fatty acids categories.

Regarding tissue distribution, the information can be rather variable, even comparing mRNA studies to each other and immunoblotting or LC–MS results with each other. The distributions may not reflect where the most physiologically important site(s) of expression are.

Catalytic efficiency (i.e., k_{cat}/K_m) is not a reliable guide to the biological importance of P450s. Even among the bacterial P450s, most are relatively slow, and only few well-studied reactions have high rates, e.g., P450 101A1 and 102A1, with camphor and fatty acid oxidations, respectively. Of these, the function of P450 102A1 is still unclear. Among the mammalian P450s using redox partners (i.e., excluding P450s 5A1 and 8A1), P450s 7A1 and 21A2 appear to be the most efficient, k_{cat}/K_m $\sim 2.5 \times 10^6$ M^{-1}/s [1775] and $\sim 10^7$ M^{-1}/s, respectively. Several of the mammalian P450s have much lower catalytic efficiencies but are clearly shown to be important in genetic studies. For instance, P450 46A1 has a k_{cat}/K_m of 500 M^{-1}/s (with cholesterol as substrate [2721, 2766]), but *Cyp46a1*$^{(-/-)}$ mice have a deficient memory phenotype [2679]. Effects of drugs on drug-metabolizing P450s can yield serious drug–drug interactions.

Some of the P450s involved in the metabolism of endogenous substrates are proving to be less selective than originally thought, e.g., P459s 7A1, 11A1, 46A1.

Following up on point vii, we are seeing more overlap between oxidations of the endogenous and xenobiotic substrates (e.g., see the Substrates and Reactions section for P450s 1B1, 11A1, and 46A1). Of course, drugs can be made to target P450s that use endogenous substrates (e.g., P450 19A1 and numerous others). There are issues in that preclinical drug candidate decisions may need to involve not only predictions of drug interactions with the major drug-metabolizing P450s (Fig. 9.1b) but also the P450s involved in oxidation of endogenous substrates, e.g., see example with P450 11A1 [1951].

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Nuclear Receptor-Mediated Regulation of Cytochrome P450 Genes

10

Saki Gotoh, Marumi Ohno, Kouichi Yoshinari, Masahiko Negishi and Kaname Kawajiri

10.1 Introduction

A half century ago, tolerance against phenobarbital, a widely used sedative to treat epilepsy, was associated with induction of drug-metabolizing enzymes in the liver endoplasmic reticulum [1]. At the same time, cytochrome P450 (CYP) was discovered and characterized as the key enzyme that metabolizes drugs [2, 3]. With these findings, P450 induction was conceptualized as the regulatory system affecting pharmacological as well as toxicological consequences of drug treatments or xenobiotic exposures. Intensive investigations were ignited to elucidate the molecular mechanism of this induction and have continued to date. Polycyclic aromatic hydrocarbons (PAHs) such as 3-methylcholanthrene (3-MC) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) emerged as the second major inducers. In the late 1970s, the aryl hydrocarbon receptor (AhR) was quickly identified as the receptor that activates its archetypal target *CYP1A1* gene [4, 5]. Another nearly 20 years would pass for phenobarbital, before the long sought after nuclear receptor constitutive androstane receptor (CAR; NR1I3) was implicated in activation of its classic target *CYP2B* gene in 1998 [6]. However, the mechanism of phenobarbital induction remained an enigma since phenobarbital does not directly bind to CAR. By the time the third edition of this text was published in 2005, PXR, NR1I2 was discovered to activate *CYP3A* genes by pregnenolone-16α-carbonitrile [7]. In addition, various other members of the nuclear receptor superfamily were also found to regulate drug-mediated activation of P450 genes including the farnesoid X receptor (FXR, NR1H4), liver X receptor (LXR, NR1H2/3), and peroxisome proliferator-activated receptors (PPARs, NR1C1/2/3). As the number of nuclear receptor-regulated P450s has increased, nuclear receptors have increasingly been placed at the center of biological processes by which cells alter their various types of metabolism from drugs/xenobiotics to endogenous substances.

These nuclear receptors, often called drug- or xenobiotic-sensing/activated nuclear receptors, were initially understood as ligand-activated transcription factors to which drugs and xenobiotics directly bind. Ligand-bound receptors directly

M. Negishi (✉) · S. Gotoh · M. Ohno
Pharmacogenetics Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, P.O. Box 12233, Mail Drop E4-07, Research Triangle Park, NC 27709, USA
e-mail: negishi@niehs.nih.gov

K. Yoshinari
Department of Molecular Toxicology, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan

K. Kawajiri
Research Institute for Clinical Oncology, Saitama Cancer Center, 818 Komuro, Ina-machi, Saitama 362-0806, Japan

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bind to specific DNA sequences within the promoter of a given target gene and activate its transcription. During the last 10 years, this simple ligand mechanism has evolved into a more complex chromatin-based mechanism to explain the specificity and diversity of nuclear receptor-mediated regulations. Intracellular localization and/or degradation of nuclear receptors also gained a place in the activation mechanism. The increase in findings that cell signaling critically regulates nuclear receptors has been observed. Nuclear receptors utilize cell signaling and either specify or diversify their regulations of *CYP* genes.

10.2 The AhR

The P450 superfamily, which appears to have diversified from a single ancestral protein to many forms over the course of biological evolution, can be found in a wide variety of life forms from animals and plants to fungi and bacteria [8]. These superfamily members are classified according to similarity of primary structures, with mammals containing 18 distinct P450 gene families that together code for approximately 50–80 individual P450 genes in any given species.

CYP1 enzymes are induced by various xenobiotics such as TCDD and this activation is regulated by the heterodimer composed of the AhR and the aryl hydrocarbon receptor nuclear translocator (ARNT) [9]. Defining the molecular mechanism of this activation has been crucial to understanding the roles of AhR in drug metabolism, chemically induced carcinogenesis, and toxicity. CYP1 enzymes are critically involved in metabolic activation of chemical carcinogens [10–12]. CYP1A1 metabolizes various species of PAHs, such as MC and benzo[*a*]pyrene, to mutagenic products. CYP1A2 metabolizes a range of drugs such as caffeine and melatonin and activates a series of aromatic amines such as 2-AAF, 2-NA, and heterocyclic amines including PhIP, IQ and Trp-1, and aflatoxin B_1 to carcinogenic products. CYP1B1 activates both PAHs and aromatic amines as well as metabolizes estrogens.

10.2.1 Ligand-Activated Transcription Factor

1. Domain Structure of AhR and ARNT

AhR and ARNT are members of a structurally related gene family with characteristic structural motifs designated as the bHLH and PAS domains [13]. The bHLH domain resides near the N-terminus of the AhR molecule from which bHLH motif mediates AhR dimerization and DNA binding, while nuclear localization (NLS) and nuclear export signals (NES) regulate intracellular localization of AhR. The PAS domain, localized in the middle of AhR, consists of two imperfect repeats of approximately 50 amino acids each (PAS A and PAS B) and constitutes an interactive surface mediating protein–protein interactions. The ligand-binding domain (LBD) overlaps in part with the PAS B region and also with the binding site for Hsp90. In addition to the PAS B domain, Hsp90 interacts with the bHLH region to mask the NLS of AhR, sequestering AhR in the cytoplasm. The C-terminal region of AhR and ARNT contains transcriptional activation domains that interact with coactivators CBP/p300 and RIP140.

2. Agonists and Antagonists

Numerous studies over the past decade categorized AhR ligands into two groups of “classical” and “nonclassical” AhR ligands [14]. “Classical” ligands are planar molecules with characteristics similar to those of PAHs and TCDD [11, 12]. On the other hand, “nonclassical” ligands have divergent physicochemical/structural characteristics [14]. Among “classic” AhR ligands, α-naphthoflavone displays both agonist and antagonist behavior in a concentration-dependent manner. Nonclassical AhR ligands include some endogenous compounds, such as indole acetic acid, indole-3-carbinol, kynurenine, lipoxin A4, and bilirubin; their AhR-binding affinities are generally weaker than those of classical ligands [14]. Among antagonists,

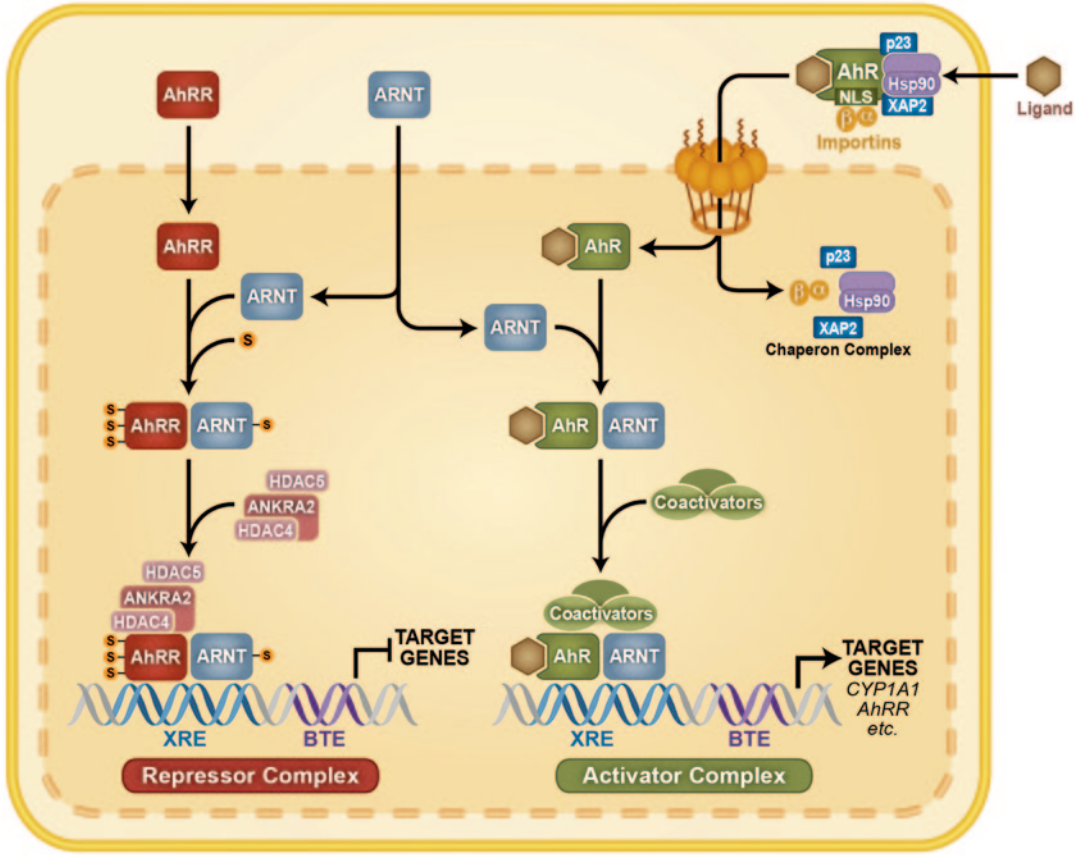

Fig. 10.1 A model for the transcriptional regulation of the AhR/ARNT activator and AhRR/ARNT repressor complexes. Unmodified ARNT forms a heterodimer with ligand-bound AhR and recruits coactivators, such as CBP/p300, to form the transcriptional activator complex. Meanwhile, ARNT forms a heterodimer with AhRR, which significantly enhances the SUMOylation of both proteins. SUMOylated AhRR recruits corepressors ANKRA2, HDAC4, and HDAC5 to form the transcriptional repressor complex. *AhRR* AhR repressor, *ARNT* aryl hydrocarbon receptor nuclear translocator

resveratrol is nondiscriminatory to a range of agonists including TCDD and antagonizes AhR–ARNT binding to the XREs for activation [15]. CH223191 antagonizes limited numbers of AhR agonists, TCDD, but not other PAH and flavonoids [16]. A purine derivative StemRegenin 1 has been shown to promote *ex vivo* expansion of human hematopoietic stem cells by antagonizing AhR [17].

10.2.2 Nuclear Import

It is well known that AhR exists in a latent state in a complex with Hsp90, XAP2 (ARA9 or AIP), and p23 in the cytoplasm (Fig. 10.1). Hsp90 binding is essential to retain AhR in the cytoplasm and this interaction is considered to mask the NLS of AhR. Overexpression of XAP2 increases accumulation of AhR in the cytosol, and the LxxLL motif of the AhR is also involved in the cytoplasmic retention of AhR through protein–protein interactions. Hsp90- and ligand-binding sites spatially overlap and ligand binding to AhR displaces Hsp90 in AhR activation [18]. This suggests a conformational change in the AhR/Hsp90 complex to expose the masked NLS of AhR that are required to facilitate interaction of the NLS with importins. It should be noted that the phosphorylation-regulated nuclear

import process may be involved [19], where a phosphorylated NLS abrogates ligand-dependent nuclear import, and dephosphorylated NLS sufficiently promotes it to interact with NLS receptors followed by Ran-GDP- and p10-mediated nuclear import. Because resistance to TCDD toxicity and loss of induction of drug oxidation activity is observed in mice carrying a mutation in the NLS, ligand-dependent nuclear translocation of AhR appears to be an important step in the induction of P450 enzymes [20].

10.2.3 Regulation of CYP1 Genes by AhR/ARNT Heterodimer

1. *Cis*-Acting DNA Element

The identification of the transcription promoter and enhancer responsible for the induction of *CYP1* was accomplished using the two assay systems that defined the ligand-dependent AhR-DNA interaction: the enhancer/promoter-driven reporter assay and the electrophoretic mobility shift assay. The regulatory DNA elements for *CYP1A1* induction by PAH, called xenobiotic-responsive element (XRE: 5′-TNGCGTG-3′, also known as DRE or AhRE), were first identified in the rat *CYP1A1* promoter [15]. AhR and ARNT preferentially bind to 5′-half-site (TNGC) and 3′-half-sites (GTG), respectively. All *CYP* genes which are activated by PAHs or TCDD carry XRE sequences within their promoter/enhancer regions, which include *CYP1A1, 1A2, 1B1, Cyp2a5, 2a8, 2s1,* and *Cyp19*.

2. Activation of *CYP1* Genes

The *CYP1* family includes three genes: *CYP1A1*, *1A2,* and *1B1*; all of which are inducible by AhR agonists. Upon ligand binding followed by nuclear translocation, AhR dissociates from the Hsp90-chaperone complex and subsequently heterodimerizes with ARNT to bind XRE sequences in the promoters of target genes (Fig. 10.1) [21, 22]. AhR binds to and enhances XREC in the approximately 1-kb upstream region of the *CYP1A1* gene. In addition, a basic transcription element (BTE), a GC box sequence immediately upstream of the transcription start site, is required for high *CYP1A1* expression; SP1 binds to BTE and synergizes AhR/XREC-mediated activation of the CYP1A1 promoter [21].

Chromatin remodeling is initiated by liganded AhR/ARNT heterodimer binding to the XREs in the enhancer region, leading to increased DNase sensitivity and creating a DNase hypersensitive site 300 bp upstream of the transcription initiation site. This binding enables the promoter to recruit coactivators such as CBP/p300, Ncoa1 NCoA1, NCoA2, NCoA3, and RIP140. RIP140 is a component of the ATP-dependent chromatin remodeling complexes with Brahma/switch 2 related gene 1 (BRG-1), p-TEFβ, and RNA elongation factors [23]. In addition, the TRAP/DRIP/ARC/Mediator complex must be recruited to the *CYP1A1* promoter to activate the *CYP1A* promoter in response to xenobiotic stress. More details for the functional formation of these complexes have been recently reviewed [22, 23].

UV-B radiation (290–320 nm) photo-produced 6-formylindolo[3,2-*b*]carbazole (FICZ) from the chromophore tryptophan. Since FICZ is a high-affinity AhR ligand, UV radiation resulted in activation of AhR, thereby nuclear translocating AhR and activating the *CYP1A1* gene in HaCaT cells. FICZ-activated AhR also simulated EGFR-ERK1/2 signaling [24]. These AhR-mediated stress responses were confirmed by in vivo studies using AhR-deficient mice.

Human *CYP1A1* and *1A2* genes are arranged in a head-to-head orientation at a distance of approximately 23 kb apart on chromosome 15. Utilizing a dual reporter vector containing the intergenic spacer region between the *CYP1A1* and *CYP1A2* genes, it was shown that XREC, previously characterized from the CYP1A1 gene, works in a bidirectional manner to activate not only *CYP1A1* but also *CYP1A2* [25]. A similar chromosomal arrangement has also been reported for mouse *Cyp1a1* and *1a2* genes on chromosome 9. The XREC was eliminated from the *CYP1a1* and *1a2* genes in the mouse genome by homologous recombination. Subsequent studies with XREC-deficient mice confirmed that XREC is sufficient for simultaneous induction of the *Cyp1a1* and *Cyp1a2* genes in response to

TCDD [26]. In addition, a novel DNA element responsive to 3-MC (XRE2) was identified in the proximal *CYP1A2* promoter, which is similar to a consensus DNA-binding sequence recognized by the LBP-1 family [27].

The *CYP1B1* gene contains XREC approximately 1 kb upstream of its transcription start site and its promoter is similarly regulated by AhR as observed with the *CYP1A1* gene. For example, as observed with the *CYP1A1* promoter, the *CYP1B1* promoter recruits histone acetylase coactivators, p300 and NCoA2 after TCDD treatment. The ATPase-dependent nucleosome remodeling factor BRG-1 is recruited to the *CYP1A1* gene upon TCDD treatment for activation; this is also the case for the *CYP1B1* gene [28]. Epigenetic modifications are known to play a significant role in transcriptional regulation of genes. CpG islands have been identified in the enhancer and promoter regions of the *CYP1A1* and *1B1* genes, and alterations in the DNA methylation status of CpG islands were compared between *CYP1A1* and *1B1* genes in various types of cancer [29]. Both genes were induced by TCDD in MCF-7 cells but *CYP1B1* was not induced in HepG2 cells. The *CYP1B1* induction deficiency in HepG2 cells is ascribable to hypermethylation of its promoter; this affects some, but not all, of the relevant TCDD-induced changes that normally occur in the gene, such as recruitment of TBP and RNA polymerase II to the promoter [30].

10.2.4 Activation of CYP2 and CYP19 Genes

CYP2S1 is unusual for a non-*CYP1* family member in that it is inducible by TCDD and is expressed at high levels in epithelial tissues that are exposed to the environment. This suggests that it may be important in metabolic activation or deactivation of procarcinogens present in the environment. Induction of mouse *Cyp2s1* is mediated by a novel complex regulatory element consisting of three overlapping XREs [31]. In addition, it is inducible by hypoxia, and this induction is mediated in part by three overlapping HREs that are contained within the trimeric XRE sequence. *CYP2A8* in Syrian hamster and *Cyp2a5* in the mouse are also inducible by AhR agonists. By analyzing the 5′-flanking region of the *CYP2A8* gene, an XRE and a novel positive regulatory element (PREX) were determined. The factor binding to PREX was identified as NF2d9 (LBP-1a), which interacts with AhR/ARNT and enhances XRE-driven transcription of the *CyPZA8* gene [32]. In addition, a putative XRE was also identified in the *Cyp2a5* promoter [33].

The *Cyp19* gene can be activated by AhR in ovarian granulosa cells. In vitro reporter gene and in vivo ChIP assays revealed that AhR cooperates with orphan nuclear receptor Ad4BP/SF-1 to activate the *Cyp19* gene. An intrinsic function of AhR appears to be to adjust ovarian estradiol concentrations by regulating the *Cyp19* gene. DMBA treatment induced ovarian *Cyp19* expression regardless of estrus cycles in female mice. This aberrant induction of the *Cyp19* may be the mechanism responsible for the toxic effects of exogenous AhR ligands as endocrine disruptors [34]. Thus, several other *CYP* genes, in addition to *CYP1* genes, can be regulated by the AhR/ARNT heterodimer.

10.2.5 Repression of AhR-Mediated CYP Activation

AhR signaling can be down-regulated by at least two independent mechanisms: one is the negative feedback inhibition of AhR by the AhR repressor (AhRR) in the nucleus and the other is proteasome degradation of AhR in the cytoplasm. AhRR was originally identified as a TCDD-induced protein and inhibited AhR signaling [35]. Newly synthesized AhRR translocates into the nucleus and forms a heterodimer with ARNT, thereby competing with XRE binding of the AhR/ARNT heterodimer and recruiting corepressors such as ANKLA2, HDAC4, and HDAC5 (Fig. 10.1). The C-terminal repression domain of AhRR has three SUMOylation sites which are conserved across vertebrate species and all three sites should be SUMOylated for complete suppressive activity [21, 35]. The AhR protein is rapidly depleted in cells in vitro following exposure to

AhR ligands, most likely after target gene activation. AhR degradation was blocked by treating with the proteasome inhibitor MG-132. Because this degradation was inhibited by leptomycin B, which is a nuclear export inhibitor, it is likely that AhR degradation occurs in the cytoplasm [36]. However, AhR degradation may also occur in the nucleus [37]. Liganded AhR forms an E3 ubiquitin ligase complex with CUL4B, DDB1, TBL3, and Rbx1/Roc1 in the nucleus and facilitates the ubiquitylation of not only AhR but also ERα, ERβ, AR, and β-catenin. This stimulated ubiquitylation is a new AhR function, which may lead to a greater understanding of the diverse biological actions induced by endogenous and exogenous AhR agonists [38].

10.2.6 Ligand-Independent Activation of AhR and Nuclear Import

When different cell lines were grown in suspension culture, AhR spontaneously translocated into the nucleus and increased CYP1A1 or CYP1B1 mRNAs in the absence of exogenous AhR ligands [39]. Cell density influenced not only the intracellular localization of AhR but also the transcriptional activation of a reporter gene driven by the XRE sequence in HaCaT cells [40]. Nuclear accumulation of AhR under low cell density conditions is also caused by phosphorylation in the NES of AhR which inhibits nuclear export of AhR. The second messenger cAMP, an endogenous mediator of hormone and neurotransmitter signaling, has also been reported to activate AhR and lead to its nuclear translocation [41]. Omeprazole induces CYP1A1 expression in an AhR-dependent manner without directly binding to AhR [42]. This suggested that cell signaling may be involved in AhR activation by omeprazole. Recently, omeprazole was found to activate the human *CYP1A1* and *CYP1A2* promoters via AhR–ARNT binding sites [43]. Utilizing species differences in the activation of AhR by omeprazole, unique amino acid residues that are required for omeprazole activation have been determined within the ligand-binding pocket of LBD [44]. It remains to be determined in future investigations whether or not omeprazole activates AhR via cell signaling and/or ligand binding.

10.2.7 Evolutionary Aspects of the AhR/ARNT System

Because gene-cloning methods have become more accessible over the past decade, information regarding AhR diversity in vertebrates has rapidly expanded. AhR is an ancient protein that was present in most major groups of animals, including deuterostomes and the two major clades of protostome invertebrates: ecdysozoans and lophotrochozoans [45]. Deuterostomes and protostomes comprise the clade of bilaterian metazoans, whose most recent common ancestor lived approximately 570 million years ago (MYA). The original function of the AhR may have contributed to a developmental regulatory gene because ancestral AhR was involved in the development of sensory structures or neurons; however, it was insensitive to the toxicity of TCDD-like compounds in early metazoans and to some extent in invertebrate species such as *C. elegans* and *Drosophila melanogaster*.

In mammals, AhR participates not only in the development of the liver, ovary, cardiovascular, and immune systems but also in regulating xenobiotic-metabolizing enzymes [21, 22]. The adaptive function of AhR may have first evolved in early vertebrates. A jawless fish, the sea lamprey, is the earliest known example of a divergent vertebrate animal (approximately 450 MYA) and its AhR has a poor ability to bind TCDD, which is consistent with the lack of *CYP1A* induction in lampreys treated with AhR ligands. The earliest divergent animals that demonstrate TCDD binding ability and AhR-mediated *CYP1A* expression were jawed vertebrates such as cartilaginous and bony fishes [45]. These jawed vertebrates diverged from human lineage approximately more than 410 and 400 MYA, respectively. The *CYP1A* gene was cloned from several teleost species, and functional XRE and AhR-mediated *CYP1A* induction by TCDD has been observed. Thus, emergence of the AhR and CYP1A functions appears to have coincided with evolution of their ability to bind HAHs and PAHs, which suggests that the adaptive function of AhR may have been an evolutionary innovation for vertebrates [45]. Although AhR is an important component of cellular defenses against exogenous and endogenous

toxicants, it would be interesting to decipher why *CYP1* induction does not utilize orphan nuclear receptors, which participate in inducible expression of families 2–4 of the P450 genes, but utilizes a different bHLH-PAS family of AhR in the evolution of vertebrate species.

10.3 The PXR

PXR, NR1I2 is a member of the nuclear receptor subfamily which also includes constitutive active/androstane receptor (CAR) and vitamin D3 receptor (VDR). PXR is primarily expressed in liver, kidney, and gastrointestinal tract. PXR was first cloned from a mouse cDNA library based on its sequence homology to other known nuclear receptors and was activated by various CYP3A inducers such as pregnenolone-16α-carbonitrile (PCN) in 1998 [8]. Orthologs of mouse PXR have been cloned from a wide range of species: mammals (including humans), birds, and fish. Subsequently, PXR knock out and humanized PXR mice were utilized to confirm the *in vivo* roles of PXR in activating the *Cyp3a* genes [46–48]. Human PXR can be activated by diverse drugs and xenobiotics. In turn, liganded PXR activates numerous genes: the *CYP2B6, CYP2B9, CYP2C8, CYP2C9, CYP3A7, and CYP2C19* genes in addition to the *CYP3A4* gene. Human CYP3A and CYP2C enzymes metabolize the majority of therapeutic drugs. Through these findings, PXR was established as the most important nuclear receptor in drug metabolism and disposition.

10.3.1 Ligand-Activated Transcription Factor

1. Domain Structure of PXR

PXR shares common structural features that are characteristic of nuclear receptors [49]: a DNA binding domain (DBD), hinge and ligand-binding domain (LBD). Ligand-independent activation function 1 (AF-1) is shortened in PXR and its function has not yet been assigned. LBD contains the ligand-dependent activation function 2 (AF-2) at its C-terminal region. PXR forms a heterodimer with retinoid X receptor α (RXRα). Upon ligand binding, the AF2 helix undergoes conformational changes, enabling PXR/RXRα to recruit coactivators, such as those found in the p160/SRC family, and transcriptionally activates target genes. Binding of antagonists altered this AF-2 conformation to inactivate PXR. Crystal structures of the PXR LBD with or without ligands have revealed that the PXR ligand-binding pocket has the ability to conform and modify its volume and shape, depending on the ligand. In addition, structural studies of PXR LBD support the notion that PXR can exist as a homodimer and activate genes [50].

2. PXR Agonists and Antagonists

PXR is a highly promiscuous receptor that binds to a variety of chemically and structurally distinct drugs, xenobiotics and endobiotics. Human PXR agonists include statins (*e.g.,* lovastatin and SR12813), hyperforin, anticancer drugs (*e.g.,* tamoxifen and taxol), antibiotics (e.g., rifampicin), natural and synthetic steroids (e.g., 5β pregnane-3,20-dione and estradiol), imidazole antifungals (*e.g.,* clotrimazole), bile acids, dietary fat-soluble vitamins, and some pesticides (*e.g.,* pyributicarb). PXR agonists exert strong species-specific effects on the activation of PXR target genes. For example, PCN is an activator of rodent PXR, not human PXR [51], whereas rifampicin activates human PXR, but not rodent PXR [52]. Unlike a large number of agonists, only a few PXR antagonists have been identified. ET-743 was first reported as a human PXR antagonist [53]. Subsequently, polychlorinated biphenyls, camptothecin, ketoconazole, fluconazole, enilconazole, sulforaphane, HIV protease inhibitor A792611, and metformin have been reported. In particular, attempts have been made to use ketoconazole for therapeutic purposes. However, the doses used were not high enough to antagonize PXR [54]. Developing safer and more high-potency ketoconazole analogs will be needed for therapeutic purposes.

3. Ligand Activation of PXR

Mouse PXR translocated from the cytoplasm into the nucleus [55]. Mouse PXR was retained in the cytoplasm by forming a complex with heat shock protein 90 (Hsp90) and cochaperone CAR cytoplasmic retention protein (CCRP) [56]. Upon ligand binding, PXR dissociated from its chaperone complex and translocated into the nucleus. Conversely, human PXR always remained in the nucleus and associated with transcriptional corepressors such as nuclear receptor corepressor 1 (NCoR1) or NCoR2/SMRT [7, 57, 58]. NCoR1 and SMRT allowed PXR to recruit HDACs to repress its basal transactivation activity [58]. Liganded PXR underwent conformational changes that led to dissociation of corepressors followed by recruitment of coactivators, such as steroid receptor coactivator 1 (SRC-1) [7] or SRC-3 and by subsequent chromatin remodeling for transcriptional activation. Liganded PXR directly binds to a DNA response element within the promoter region of its target genes as a heterodimer with retinoid X receptor α (RXRα; Fig. 10.2). Other reported PXR co-regulators include p300/CBP, RIP140 [59], peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) [60], hepatocyte-enriched nuclear factor 4α (HNF4α) [61], and protein arginine methyltransferase 1 (PRMT1) [62].

4. Cell Signaling-Mediated Regulation of PXR

Hepatic drug-inducible P450 gene expression has been well connected with protein kinase signaling pathways. The cyclic AMP-dependent protein kinase (PKA) signaling effectively phosphorylated PXR both *in vivo* and *in vitro* and modulated its activity in a species-specific manner [63, 64]. In mouse hepatocytes, activation of PKA signaling increased PXR-mediated gene activation, while PKA repressed it in both human and rat hepatocytes. Protein kinase C (PKC) signaling also phosphorylated PXR and attenuated the transcriptional activity of PXR by increasing its interaction with NCoR and abolishing the ligand-dependent interaction with SRC-1 [57]. Lin *et al*. reported that cyclin-dependent kinase 2 (Cdk2) directly phosphorylated human PXR, most likely at residue serine 350. Activation of Cdk2 led to inhibition of PXR-mediated CYP3A4 expression [65]. Furthermore, a recent study has shown that p70 S6K, a downstream kinase in the PI3K/Akt signaling pathway, phosphorylated PXR and negatively regulated the transcriptional activity of PXR. p70 S6K appeared to phosphorylate threonine 57 of PXR to repress activity [66]. Thus, regulation of PXR activity by phosphorylation has come to light and should warrant further investigations.

10.3.2 Regulation of CYP Genes by PXR

1. *Cis*-Acting DNA Elements

PXR binds to the AGGTCA-like direct repeats DR-3, DR-4, or everted repeats ER-6 and ER-8. The human *CYP3A4* promoter contains a proximal ER-6 and a distal xenobiotic-responsive enhancer module (XREM) that consists of DR-3 and ER-6 [67, 68]. DR-3 type XREs are present in the proximal promoters of the rat *CYP3A23* and *CYP3A2* genes [8, 69]. In addition to these *CYP3A* promoters, the PXR has been shown to bind to DR-4 and ER-8 response elements within the *CYP2B* promoters [70, 71]. Since CAR also binds to these response elements, both PXR and CAR regulate the same genes in response to their activators [70].

2. Activation of *CYP2C* Genes by PXR

The human CYP2C subfamily consists of four members, CYP2C8, CYP2C9, CYP2C19, and CYP2C18. PXR response elements have been identified within their promoters. In the *CYP2C9* promoter, DR4 and DR5 were present and named CAR/PXR-RE. The CYP2C19 promoter also contains CAR/PXR-RE. The *CYP2C8* promoter includes two DR4s [72–74]. These elements were bound and activated by both PXR and CAR in gel shift and reporter assays, respectively. Thus,

human *CYP2C* genes can be regulated by PXR and/or CAR activators. PXR required HNF4α to fully activate the *CYP2C* promoter, which will be detailed in the section "Cross Talk".

3. Activation of *CYP3A* Genes by PXR

PXR is a master regulator for expression of the CYP3A enzyme that catalyzes the metabolism of more than 50 % of all clinically used drugs. Upon ligand binding followed by dissociation from corepressors such as NCoR1 and SMRT, the PXR-RXRα heterodimer binds to both the distal XREM and proximal ER6 within the human *CYP3A4* promoter. Subsequent to these bindings, DNA looping occurs to bring XREM and ER6 in close proximity to assemble a pre-initiation complex with RNA polymerase II (Fig. 10.2). In response to rifampicin treatment, the *CYP3A4* gene undergoes epigenetic modifications; the *CYP3A4* promoter recruits PRMT1 which directly interacts with PXR to methylate arginine 3 of histone H4 and activates transcription [62]. In addition to its gene levels, PXR can also be regulated at mRNA levels by miRNAs; miR-148 facilitated degradation of PXR mRNA and/or reduced translation to repress CYP3A4 expression [75]. The miR-27b directly repressed CYP3A4 mRNA [76]. PXR requires HNF4α to activate the *CYP3A4* promoter, which will be detailed in the section "Cross Talk".

10.3.3 The CYP7A Gene in Bile Acid Homeostasis

Bile acids are the end products of hepatic cholesterol catabolism and play essential roles in eliminating cholesterol from the body. However, pathophysiological accumulation of bile acids elicits cytotoxicity and can lead to cholestasis in livers. PXR plays a critical role in bile acid detoxification, by regulating bile acid biosynthesis, transport and metabolism. Studies in PXR knock out and humanized PXR mice revealed that PXR reduces secondary bile acid lithocholic acid (LCA)-induced liver toxicity [77]. PXR regulates expression of CYP7A1, the rate-limiting enzyme of bile acid synthesis. Activated PXR suppresses HNF4α-mediated *CYP7A1* activation by inhibiting PGC-1α [60]. LCA and its direct metabolite 3-keto-LCA are efficacious activators of both mouse and human PXR; thus, activating PXR can increase bile acid clearance by inducing CYP3A, bile acid transporters, organic anion-transporting polypeptide (OATP) 2, and multidrug resistance-associated protein 2 (MRP2) [48]. Collectively, PXR serves as a pathophysiological sensor of bile acids to maintain bile acid homeostasis both by decreasing bile acid synthesis and increasing metabolism and excretion.

10.3.4 PXR in Inflammation

Inflammation and infection reduced hepatic expression of drug-metabolizing CYP enzymes. Activation of nuclear factor-kappa B (NF-κB) by lipopolysaccharide or tumor necrosis factor α (TNFα) interfered with PXR/RXRα binding to the *CYP3A4* promoter, thus suppressing transcription and CYP3A enzyme activity [78]. In turn, exposures to PXR-activating xenobiotics such as insecticides and pesticides are known to adversely affect immune functions. However, PXR activators such as rifampicin have long been known to suppress humoral and cellular immunological responses in liver cells. Recent studies demonstrated that commonly used drugs activate PXR to inhibit NF-κB activity. Expressions of typical NF-κB target genes, such as cyclooxygenase-2 and TNFα, are substantially elevated in multiple tissues, particularly in small bowel inflammation in PXR knockout mice [79]. This elevation could be caused by loss of negative regulation of NF-κB activity by PXR activation. In addition, SUMOylation of PXR appeared to play an important role in repression of inflammatory responses. In response to inflammation, liganded PXR was SUMOylated by conjugating SUMO3 chains and SUMOylated PXR repressed expression of NF-κB target genes and immune responses [80]. Therefore, via PXR, drugs like rifampicin attenuate inflammation, while inflammation represses drug metabolism.

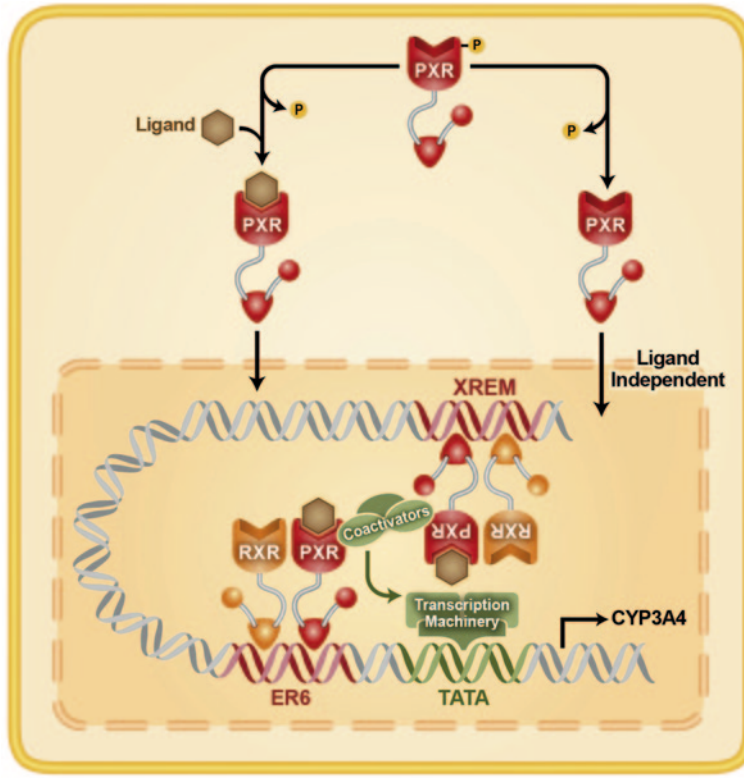

Fig. 10.2 A model for PXR-mediated regulation. PXR can potentially undergo various types of posttranslational modifications [171]. These include possible phosphorylation sites (such as Thr57, Thr248, Tyr249, Thr290, and Thr Ser 350) which studies suggest are of regulatory significances. In the case of Thr290, phosphorylated PXR is retained in the cytoplasm. Ligand-binding translocates nonphosphorylated PXR into the nucleus. PXR–RXR heterodimer then binds to the response elements (distal XREM and proximal ER6 that can work independently), which may alter chromatin structure for promoter activation. In addition, it is known that nonphosphorylated PXR is capable of undergoing ligand-independent nuclear translocation for gene activation. *PXR* pregnane X receptor, *RXR* retinoid X receptor. [172]

10.4 The CAR

The constitutive/active androstane receptor CAR, originally named MB67, was first cloned as a nuclear receptor that constitutively activates the retinoic acid response element in cell-based transfection assays [81]. Then, from 1998 to 1999, the function of CAR relative to the induction of *CRP* genes as a phenobarbital activated nuclear receptor was established [7, 82, 83]. Subsequently, the *in vivo* roles of CAR in induction were confirmed using CAR KO mice [84, 85]. One of the major interests of the past half century has been to define the molecular mechanism of phenobarbital induction. This mechanism is now delineated; phenobarbital antagonizes epidermal growth factor receptor signaling to indirectly activate CAR (Fig. 10.3). Readers are advised to refer to a recent review that is more oriented towards aspects of ligand activation [86].

10.4.1 PBREM and CAR

The quest to identify CAR began by looking for the phenobarbital responsive DNA sequence within

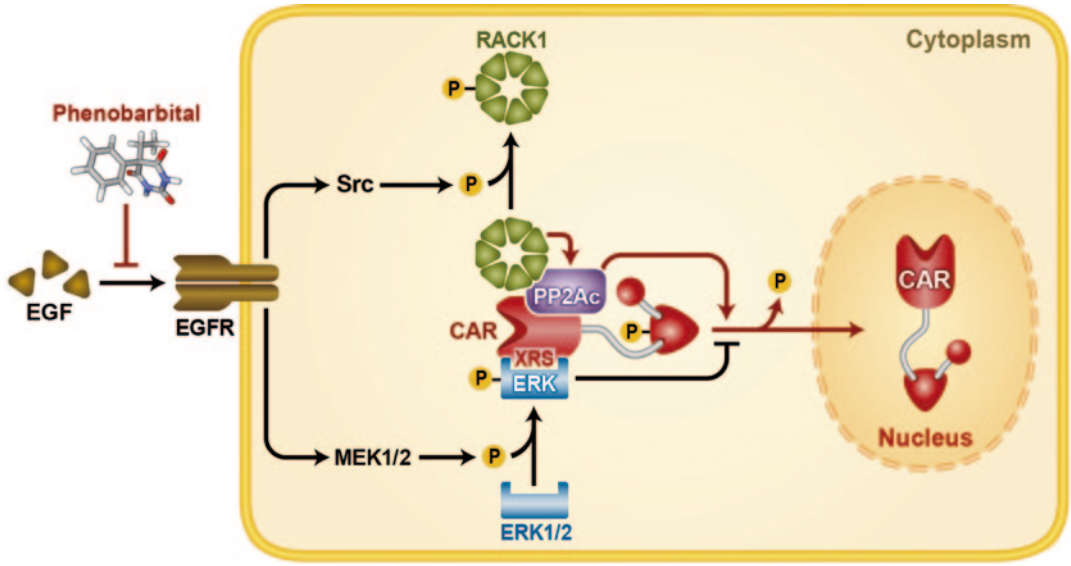

Fig. 10.3 A model for cell signaling-mediated mechanism of CAR activation. Phenobarbital (*PB*) directly binds to EGFR and antagonizes the EGF–EGFR signaling cascade to facilitate RACK1 dephosphorylation. In the presence of nonphosphorylated RACK1, PP2Ac dephosphorylates CAR in the cytoplasm. Nonphosphorylated CAR then translocates into the nucleus to activate target genes. A more detailed mechanism by which XRS-ERK1/2 regulates RACK1-PP2C to repress CAR dephosphorylation remains the key feature to be resolved in future investigations. *EGFR* epidermal growth factor receptor, *CAR* constitutive androstane receptor

the *CYP2B* promoters. Anderson and his coworkers first determined a phenobarbital responsive DNA sequence within the rat *CYP2B2* promoter, named PBRE [87]. This PBRE sequence was further minimized to the 51-bp phenobarbital responsive enhancer module (PBREM) within the mouse *Cyp2b10* promoter [88]. PBREM can be activated by a myriad of phenobarbital-type inducers and is conserved in *CYP2B* genes from mouse to human [88, 82]. PBREM or related DNA sequences are also present in the other *CYP* genes as well as in genes that encode transferases and transporters: *CYP1A, CYP2B, CYP2C, CYP3A, GST, UGT* and *SULT* [89, 90]. Negishi's laboratory identified CAR as a nuclear receptor that binds to the DR4 motifs within PBREM and activates it [6]. Utilizing an oligonucleotide as an affinity ligand, proteins were purified from liver nuclear extracts prepared from saline- or phenobarbital-treated mice. Western blot analysis revealed that nuclear receptors RXR and CAR increased their binding to DR4 motif after phenobarbital treatment. Subsequent gel-shift and reporter analyses confirmed that a RXR-CAR heterodimer binds to the DR4 motif and activates PBREM in cell-based reporter assays [6]. Neither phenobarbital nor the known potent ligand (1,4-bis[2- (3,5-dichloropyridyloxy)]benzene) (TCPOBOP) was able to activate the *Cyp2b10* gene in the livers of CAR KO mice [84, 85, 90]. Microarray analysis revealed CAR-dependent induction of CYP2B10, 3A11, 2D9, 2D10, 2J5, and 2F2 in PB-treated mouse liver [85]. In addition to mammal, the chicken xenobiotic receptor (CXR, homologue of CAR and PXR) activated phenobarbital response unit (PBRU) within the chicken *CYP2H* gene [91].

10.4.2 Structural Features of CAR

CAR, unlike other nuclear receptors that are activated by binding of a given agonist, is constitutively activated in cell-based assays. Adding a peptide to the C-terminus repressed this constitutive activity of mouse CAR [92]. In another study, mutation of Thr176 on α-helix 3 or Thr350 on α-helix 3 (AF2 domain) abolished the constitutive activity and conferred ligand activation capability to mouse CAR [93]. As to the mechanism, Thr176 formed a hydrogen bond with Thr350 in the mouse CAR model structure, which may constrain the AF2 domain to an active conformation. However, Thr176 is conserved but Thr350 is replaced with Met340 in human CAR. The X-ray structures of the ligand-binding domains of human and mouse CARs have been resolved [94]. The overall structures of CAR LBD are similar to those of other nuclear hormone receptors. In these CAR structures, α-helix 12 (AF2 helix) tightly packs with α-helix 3, thereby constraining CAR in the active conformation to interact with coactivators such as SRC1, TIF2 or RAC3 [95]. This conformation may be stabilized by hydrogen bond interactions between Lys194 (in human) or Lys205 (in mouse) with their C-terminal carboxyl group [94]. Androstanol, a reverse agonist, in the mouse CAR structure kinks the linker between α-helices 10 and 11 to relax α-helix 12 into the inactivating conformation [94].

10.4.3 Cell Signaling that Regulates CAR

CAR is constitutively activated in transformed cells such as HepG2 cells. This constitutive activity is suppressed in order to acquire *in vivo* responsiveness in organs such as liver. For this, CAR is retained in the cytoplasm [83]. Treatment with CAR activators translocates CAR from the cytoplasm into the nucleus for activation. CAR can be activated either directly or indirectly. Direct activation by ligands exhibits species differences with TCPOBOP and CITCO (6-(4-chlorophenyl)-imidazo[2,1-b] [1,3] thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime) preferentially activating mouse and human CAR, respectively [96, 97]. On the other hand, phenobarbital, an indirect activator, can equally activate mouse, rat, and human CAR. This cross species activation indicates that the cell signaling-mediated regulatory mechanism should be conserved in mouse as well as humans.

1. Dephosphorylation of Threonine 38

Involvement of cell signaling in CAR activation was first suggested by the finding that okadaic acid, a protein phosphatase inhibitor, repressed phenobarbital-induced nuclear CAR accumulation and increase of CYP2B10 mRNA in mouse primary hepatocytes [83, 98]. The CAR residue that is dephosphorylated after phenobarbital treatment is Thr38 in human CAR and Thr48 in mouse CAR and the protein phosphatase that dephosphorylates this site is protein phosphatase 2A (PP2A) [99]. Hereafter, Thr38 will be used to describe phosphorylation for both human and mouse CAR for practical purpose. A phosphorylated peptide antibody (αP-T38) was utilized to detect phosphorylated CAR at Thr38 in mouse primary hepatocytes. Phosphorylated CAR is retained in the cytoplasm in mouse hepatocytes. Phenobarbital treatment triggered dephosphorylation and resultant nonphosphorylated CAR translocated into the nucleus [99, 100]. The YFP-tagged CAR Thr38Ala (nonphospho-mimicking) mutant, directly expressed in the mouse livers, spontaneously translocated into the nucleus before treatment, while the phospho-mimicking Thr38Asp mutant was retained in the cytoplasm even after phenobarbital treatment [99]. In reporter and gel shift assays, the Thr38Asp mutant neither bound to PBREM nor activated it [99]. Thus, phosphorylation of the single Thr38 site both inactivates *trans-activity* of CAR and retains it in the cytoplasm. Dephosphorylation of Thr38 is the underlying mechanism that activates CAR. This mechanism of CAR activation is conserved, as a recent report just confirmed dephos-

phorylation of human CAR in human primary hepatocytes after phenobarbital treatment [101].

2. Protein Phosphatase 2A and RACK1

Since okadaic acid preferentially strongly inhibits PP2A over other protein phosphatases, repression of phenobarbital-induced nuclear CAR accumulation finger indicated PP2A [83, 98]. In *in vitro* dephosphorylation assays using recombinant CAR phosphorylated at Thr38 as a substrate, PP2A was not able to dephosphorylate Thr38. However, adding receptor for activated kinase C 1 (RACK1) enabled PP2A to dephosphorylate Thr38 [102]. Thus, PP2A was the enzyme that dephosphorylates Thr38, in which RACK1 functions as the regulatory subunit that activates the core enzyme. Knock down of either the PP2A catalytic subunit or RACK1 by siRNAs abolished phenobarbital-induced Thr38 dephosphorylation as well as increased CYP2B10 mRNA [102]. RACK1 can be phosphorylated at Tyr52; only nonphosphorylated RACK1 enabled PP2A to dephosphorylate Thr38 in *in vitro* assays [102]. If phenobarbital elicits a signal to dephosphorylate Tyr52, RACK1 can be the regulatory mediator between phenobarbital and CAR activation.

3. EGFR as the Phenobarbital Receptor

Endogenous stimuli such as growth hormones and insulin have long been known to repress phenobarbital induction of P450 genes. It has also been known for a long time that phenobarbital treatment antagonizes membrane signaling mediated by the epidermal growth factor receptor (EGFR) and insulin receptor. Bauer et al. were the first to demonstrate that growth factor or EGF represses CAR-mediated activation of the PBREM reporter gene in rat primary hepatocytes [103]. Given this link between EGF and CAR activation, Negishi's laboratory defined EGFR as the phenobarbital binding site through which phenobarbital initiates the signal to dephosphorylate Tyr52 of RACK1 for CAR activation [102]. *In vitro* binding assays utilizing either direct isothermal titration calorimetry or indirect binding competition between phenobarbital and EGF confirmed that phenobarbital binds to EGFR with K_d values around 10 μM [102]. Treatment with phenobarbital within the range of these K_d values repressed EGF-activated phosphorylation of EGFR in mouse primary hepatocytes. Concomitant with this repression, Tyr52 of RACK1 was dephosphorylated. The resultant nonphosphorylated RACK1 enabled PP2A to dephosphorylate CAR for activation [102]. Thus, the underlying mechanism for phenobarbital induction proceeds by the following steps: (1) phenobarbital binding to EGFR, (2) dephosphorylation of RACK1, (3) dephosphorylation of CAR by PP2A-RACK1, and (4) nuclear translocation of nonphosphorylated CAR. As to how general this mechanism is, two questions should be answered in future studies: whether or not other indirect CAR activators utilize this EGFR-RACK1-PP2A mechanism to activate CAR and how CAR ligands such as TCPOBOP and CITCO activate CAR. While these ligands promote CAR binding to PBREM and activate it, as observed with phenobarbital, as long as CAR is phosphorylated they are unable to do so [99].

10.4.4 XRS, an Intramolecular Peptide Signal Peptide of CAR

Upon EGF activation, EGFR triggers at least two signals; Src kinase pathway is one and MEK–ERK pathway is another. Inactivation of the MEK–ERK signal in a growth hormone releasing hormone knockout mouse resulted in the repression of CYP2B in liver [104]. The MEK–ERK pathway was, in fact, the first to be associated with CAR activation [105]. EGF treatment repressed TCPOBOP-induced nuclear CAR accumulation in mouse primary hepatocytes, while inhibition of MEK by U0126 spontaneously translocated CAR into the nucleus and activated the *Cyp2b10* gene [105]. Subsequently, it was found that U0126 treatment dephosphorylates Thr38 of CAR [100]. In this dephosphorylation, a leucine-rich peptide (^{313}LXXLXXL^{319}) near

the C-terminus of CAR engaged as the intramolecular signal peptide to transduce MEK–ERK signaling onto CAR dephosphorylation. This peptide, called xenobiotic response signal (XRS), was first characterized as the peptide motif that regulates nuclear translocation of CAR in mouse liver [106]. XRS bound to active ERK and dissociated inactive ERK when this signaling was attenuated by U0126, resulting in dephosphorylation of Thr38 [100]. Thus, by antagonizing EGFR, phenobarbital elicits at least two different signals; one that is directly transduced to CAR via XRS and another that dephosphorylates RACK1 to activate PP2A. The molecular mechanism which integrates these two signals to converge onto CAR for dephosphorylation must be defined in future investigations.

10.4.5 Other Cell Signaling and Signal Molecules

1. AMPK Signaling

Wolf and his associates first developed HepG2-derived WGA cells in which phenobarbital treatment induced CYP2B6 mRNA and suggested that AMP-activated protein kinase (AMPK) may mediate this activation [107]. Meyer's laboratory followed up on the AMPK scenario and continued to establish it as a signal mechanism for phenobarbital induction. Although phenobarbital-induced AMPK activation and CYP2H1 mRNA increase occurred in chicken primary hepatocytes or LMH cells, it was not shown whether or not AMPK activated chicken nuclear receptor CXR after phenobarbital treatment [108]. Studies utilizing liver specific AMPK subunits α1/α2 KO mice demonstrated that basal expression of CYP2B10 mRNA was greatly increased by 100-fold in the livers of KO mice compared with that in wild-type livers. As a result, phenobarbital-induced increases of this mRNA were severely diminished to only a two- to threefold increase in KO livers compared with 200- to 300-fold in wild-type livers [109]. Moreover, phenobarbital treatment normally translocated CAR from the cytoplasm to the nucleus in AMPKα1/α2 KO primary hepatocytes. Thus, these studies did not directly connect AMPK signaling with CAR for phenobarbital induction, although AMPK may still regulate basal expression of CYP2B10 mRNA. Studies utilizing AMPK activators (e.g., AICAR) or inhibitors (e.g., 8-bromo-AMP) resulted in confusion and provided no consensus as to whether or not and how phenobarbital utilizes AMPK to activate CAR [108, 110]. Metformin is a drug widely used to treat type 2 diabetes patients. Metformin treatment alone activated AMPK kinase but neither nuclear translocated CAR nor induced CYP2B6 mRNA in human primary hepatocytes. Furthermore, metformin co-repressed phenobarbital- or CTICO-induced nuclear translocation and increased CYP2B mRNA in human primary hepatocytes [101]. However, this study presented no direct evidence that metformin repressed nuclear CAR translocation via AMPK activation. Thus, the AMPK scenario for phenobarbital induction remains elusive and may not be conserved across species.

2. Glucocorticoid Signaling

Phenobarbital treatment induced CYP2B1/2 mRNA only weakly in rat hepatocytes in the absence of glucocorticoid. A functional glucocorticoid response element was present in both rat and mouse *CYP2B* promoters [111]. However, phenobarbital treatment induced CYP2B mRNA in the livers of glucocorticoid receptor (GR) KO mice [112], while dexamethasone treatment induced this mRNA in those of CAR KO mice [111, 113]. Thus, CAR does not require GR to activate the *CYP2B* promoter; these two nuclear receptors independently regulate the *CYP2B* promoter. GR has been suggested to bind to the -4477/-4410 region of human *CAR* promoter and activate it in human primary hepatocytes [114, 115]. However, this GR-mediated activation was not observed in rat primary hepatocytes [116]. The modulator roles of GR on CAR expression appear to be complex and not fully understood and may not be conserved across species.

3. Chaperones, Co-Chaperones and Proteasome Signals

CAR forms a complex with a cochaperon cytoplasmic CAR retention protein (CCRP/DNAJC7) and HSP90 in the cytoplasm of HepG2 cells and is co-localized with tubulin in the cytoplasm of mouse liver cells [117]. TCPOBOP treatment recruited HSP70 to this CAR-CCRP complex and facilitated ubiquitination of CCRP. Ubiquitinated CCRP appeared to degrade, thereby releasing CAR for nuclear translocation [118]. Proteasomal inhibition by MG132 repressed phenobarbital-induced nuclear CAR accumulation and CYP2B6 mRNA elevation in human primary hepatocytes [119].

10.4.6 Regulations in the Nucleus

1. Chromatin Remodeling

Co-treatment with TCPOBOP and okadaic acid synergized induction of CYP2B6 mRNA in mouse CAR-expressing HepG2 cells [120]. This synergistic activation of the *CYP2B6* promoter was regulated by two distinct DNA sequences, a distal PBREM (−1733/−1683) and a proximal $OARE_{KI}$ (−236/−217) within the promoter [120, 121]. Two response factors, cohesin protein SMC1 and early growth response 1 (EGR1) were shown to bind to the $OARE_{KI}$. In response to protein kinase C signaling, EGR1 binds to $OARE_{KI}$ and loops the CAR-bound PBREM towards the $OARE_{KI}$, thereby synergizing activation of the CYP2B6 promoter by TCPOBOP [122]. SMC1 binding may stabilize this looping structure of the promoter, as this kind of function was recently suggested for cohesin [123]. In a study of Inoue et al., HNF4 α constitutively bound to the OARE during synergistic activation. On the other hand, another study revealed that liver-enriched HNF4 α and C/EBPα bound to both distal enhancers PBREM and XREM (−8597/−8495) and proximal promoter in order to fully activate the *CYP2B6* promoter in human primary hepatocytes [124].

Med25 is one of the mediator proteins and constitutively binds to the *CYP2C9* promoter in HepG2 cells as well as induces CYP2C9 mRNA in human primary hepatocytes [125]. Med25 binding appeared to loop the CAR-binding site toward the proximal promoter, thereby facilitating recruitment of RNA polymerase II to the promoter. Thus, CAR-mediated activation of the *CYP2B6* promoter appears to involve chromatin remodeling.

2. p38 MAPK and CaMK

Ligand activation of CAR resulted in an effective induction of CYP2B6 in human primary hepatocytes but not in HepG2 cells. This effectiveness correlated with high levels of phosphorylated p38 MAPK in hepatocytes; treatment with p38 MAPK activator restored the effective induction in HepG2 cells [126]. Thus, ligand binding alone does not appear to be sufficient for CAR to *trans*-activate its target genes. Intriguingly, CAR required p38 MAPK in the activation of only one set of genes including *CYP2B6, CYP2A7,* and *CYP2C9,* but not *CYP3A4* and *UGT1A1* genes. Treatment with a Ca^{2+}-calmodulin-dependent protein kinase (CaMK) inhibitor KN62 did not affect TCPOBOP-induced nuclear CAR accumulation in mouse primary hepatocytes, but repressed the activation of *Cyp2b10* gene [127]. Similar to the CaMK inhibitor, PPAR ligands (Wy-14643 and fibrates) and peripheral benzodiazepine receptor ligand (PK11195) induced nuclear CAR accumulation but did not activate the *CYP2B* genes [128, 129]. These observations suggest that additional nuclear signaling is essential to regulate CAR properly.

10.5 Other NRs and Cross Talk

In addition to AHR, CAR and PXR, other nuclear receptors, both constitutively active and ligand-activated ones, are known to regulate P450 expression. These nuclear receptors utilize not only the mechanism of direct transcriptional regulation but also cross talk with AhR, CAR, and PXR.

10.5.1 The HNF4α

HNF4 α (NR2A1) is a liver-enriched nuclear receptor that plays essential roles in liver development and function. A number of *CYP* genes are also repressed the liver of liver-specific HNF4 α KO mice. Utilizing other gene knockdown technologies, a recombinant adenovirus expressing antisense RNA was used to infect human primary hepatocytes [130]. In the resultant HNF4 α knocked down hepatocytes, mRNA levels of CYP3A4, CYP3A5, and CYP2A6 were greatly reduced and those of CYP2B6, CYP2C9, and CYP2D6 were moderately reduced. On the other hand, CYP2E1 mRNA levels remained constant. A recombinant adenovirus expressing HNF4 α siRNA infected human primary hepatocytes confirmed that the overall changes in CYP mRNA levels were similar to those obtained with antisense RNA [131]. In addition, CYP1A2, CYP2C8 and CYP2C19 mRNAs reduced their levels, while CYP1A1 and CYP2J2 mRNA levels remained constant. In addition to *CYP* genes, transferase and transporter genes (e.g., *UGT1A1, SULT2A1, ABCB11* and *OCT1*) were repressed in HNF4 α knocked down hepatocytes [131]. Thus, HNF4 α appears to regulate the basal expression of these genes involved in drug metabolism and disposition. However, CAR and PXR mRNA levels were also reduced in HNF4α knocked down hepatocytes, thus suggesting the possibility that HNF4α also regulates those *CYP* genes indirectly via CAR and/or PXR. Analysis of 20 human liver samples demonstrated that HNF4 α mRNA levels correlate with those of CAR and PXR as well as with *CYP* genes [132].

1. The *CYP2C* Genes

There are four human CYP2C enzymes; CYP2C8, CYP2C9, CYP2C18, and CYP2C19, among which CYP2C9 and CYP2C19 play critical roles in the metabolism of therapeutics. CYP2C9 expression levels are higher than those of CYP2C19 in human livers and this difference may result from preferential regulation of *CYP2C9* by HNF4α [133]. Two identical DR1 motifs were characterized in the proximal promoter regions of the *CYP2C9* and *CYP2C19* genes. However, co-expressed HNF4 α transactivated the *CYP2C9* promoter, but not *CYP2C19,* in human hepatocarcinoma FLC7 cells. Moreover, ChIP assays demonstrated that HNF4α bound to the *CYP2C9* promoter but not to the *CYP2C19* promoter in human liver samples. On the other hand, there is a report that HNF4α transactivates *CYP2C19* through these DR1 motifs in both reporter and gel shift assays [134]. At present, the reason for the differential regulation between *CYP2C9* and *CYP2C19* remains unclear. The *CYP2C8* promoter also contains the DR1 motif and was activated by co-expression of HNF4 α [73].

2. The *CYP3A* Genes

Both the rat *CYP3A2* and *CYP3A1*/*CYP3A23* genes contain HNF4 α-binding motifs in their proximal promoters and were activated by HNF4α in co-transfection assays [135, 136]. HNF4α also regulated basal expression of mouse *Cyp3a* genes in the liver; CYP3A11/13/16 mRNAs were not detected in the liver of HNF4 α-deficient mice [61]. Moreover, a DR1 motif was characterized as a functional HNF4 α-binding site in the distal region (−1580/−1568) of the *Cyp3a11* promoter [137]. This HNF4 α-mediated *Cyp3a11* activation was suppressed in mouse livers via the sterol-responsive transcription factor SREBP-2, which inhibited PGC1 α binding to HNF4α on the promoter [137]. The two different HNF4α-binding motifs have been identified in the constitutive liver enhancer module of *CYP3A4*, CLEM4 (−10.5/−11.4 Kbp) and the *CYP3A4* enhancer module called XREM (−7.2/−7.8 Kbp) [61, 138]. HNF4 α synergistically activated PXR- and CAR-mediated transcription of the *CYP3A4* gene via XREM [61].

3. Other *CYP* Genes: *CYP2A6* and *CYP2D6*

The *CYP2A6* gene was directly regulated by HNF4α; a DR1 motif as well as an Oct-1 or C/EBP α binding motif were identified in the proximal promoter [139]. Results obtained by reporter assays in HepG2 cells and mouse livers

demonstrated that HNF42 α cooperates with these two factors to activate the *CYP2A6* promoter. Studies by Jover et al. [130] and Kamiyama et al. [131] have demonstrated that HNF4 α is involved in the basal expression of *CYP2D6* in human hepatocytes. This is consistent with the previous finding that the proximal *CYP2D6* promoter (up to −392 bp) was transactivated by co-expressed HNF4 α in COS-7 cells [140]. Thus, HNF4 α is involved in both basal and xenobiotic-responsive expressions of a number of *CYP* genes in liver in cooperation with or without other transcription factors.

10.5.2 The PPARα

PPAR α (NR1C1) is highly expressed in the livers of rodents, and to lesser extent humans, and plays a crucial role in hepatic lipid metabolism. PPAR α, activated by hypolipidemic fibrates, a variety of fatty acids and their derivatives, regulates *CYP4A* genes. Since CYP4A enzymes catalyze ω and ω-1 oxidation of fatty acids, their induction may constitute a part of the regulatory mechanism in lipid homeostasis. In addition, CYP2B was induced by fibrates in rat livers and primary hepatocytes [141, 142]. However, since fibrates can also activate mouse CAR in cell-based reporter assays [143], it remains elusive as to whether it is PPAR α or CAR that directly activates *CYP2B* genes in response to fibrates. Alternatively, fibrates could induce CAR through PPARα to activate *CYP2B* genes as indicated by the observation that fibrate treatment increased CAR mRNA and protein as well as CYP2B10 mRNA in rat primary hepatocytes [144]. Other studies with human cells suggested that PPAR α indirectly regulates the *CYP1A1* gene by either up- or down-regulation through AHR expression after fibrate treatment [145, 146].

CYP2Cs were also regulated by PPAR α in rat livers. Treatment of rats with the synthetic PPAR α ligand, WY-14,643 or gemfibrozil, decreased CYP2C11 in males and CYP2C12 in females [147]. CYP2C7 was repressed in the liver of both sexes [148]. With regard to the *CYP3A4* gene, PPAR α directly activated its transcription [149, 150]. SNP analysis of healthy human liver bank samples identified SNP rs4253728 in the *PPAR α* gene as associated with decreased atorvastatin 2-hydroxylation activity which is catalyzed by CYP3A4. Moreover, functional PPAR α binding motifs were determined in the distal *CYP3A4* promoter by multiple binding and reporter assays.

10.5.3 The LXR

Liver X receptor (LXR, NR1H) includes two isoforms. LXR α is primarily expressed in liver, intestine and macrophages, while LXRβ is ubiquitously expressed. LXRs can be activated by oxysterols such as 4β-hydroxycholesterol, 24(*S*)-hydroxycholesterol (24-HC) and 24(*S*),25-epoxycholesterol. Synthetic agonists such as GW3965 and T0901317 also activate LXRs. LXR–RXR heterodimer binds to a DR4 motif to activate its target genes. Target genes include *CYP7A1* and those involved with lipid homeostasis, such as *SREBP1*, *ABCA1, ABCG5* and *ABCG8*.

10.5.4 PXR or CAR Cross Talk

1. With LXR

Yoshinari's laboratory has defined a unique mechanism by which LXRα either activates or represses the human *CYP3A4* gene in human primary hepatocytes [151]. LXRα binds to known PXR-binding motifs dNR1 and eNR3A4 within the distal promoter termed XREM to activate it. This activation was greatly attenuated by siRNA-mediated LXR α knocked down in HepaRG cells. As expected, by sharing the same binding motifs, LXR α and PXR cross talk to regulate the *CYP3A4* gene. The ability of rifampicin, a PXR agonist, to activate the *CYP3A4* gene was weakened when human primary hepatocytes or HepaRG cells were co-treated with LXR α agonists. In addition, rifampicin treatment was more effective in inducing *CYP3A4* mRNA than an LXR α / PXR dual agonist T0901317, although T0901317 is a

stronger PXR agonist than rifampicin. These results suggest that a given compound's ability to induce CYP3A4 in human hepatocytes does not necessarily reflect its ability to activate PXR in cell-based reporter assays.

24(*S*),25-Epoxycholesterol treatment increased *CYP3A* mRNA levels in rat primary hepatocytes [152]. However, studies with primary hepatocytes from LXR KO or PXR KO mice showed that PXR, but not LXR, regulates this induction [152]. In human hepatocytes 24(*S*),25-epoxycholesterol did not increase *CYP3A4* mRNA levels [153].

Cross talk also occurs with CAR. Increases in *CYP2B6* mRNA levels in HepaRG cells after CITCO treatment were reduced by co-treatment with the LXRα agonist GW3965 [151]. Cross talk was also confirmed in mouse livers in vivo. Mice that lack both LXRα and LXRβ show increased basal levels of *Cyp2b10* and *Cyp3a11* mRNA in their livers [154]. On the other hand, induction of *Cyp2b10* and *Cyp3a11* mRNA by TCPOBOP treatment was attenuated in the livers of mice that overexpressed a dominant active form of LXRα [154]. In ChIP assays, LXRα competed with CAR for binding to the *Cyp2b10* promoter, thereby repressing induction. Another study with LXRα KO mice demonstrated that LXRα regulates *Cyp2b10* and *Cyp3a11* genes differentially in response to diets, when these mice are fed standard or cholesterol-containing food [155].

2. With AHR

LXRα directly regulated *CYP1A1* and *CYP1A2* genes [156, 157]. In response to the LXRα agonist T0901317, LXRα *trans*-activated the *CYP1A1* promoter in cell-based reporter assays in HepG2 cells and bound the -446/-607 region in ChIP assays [156]. A DR4-type motif was found within this region, to which LXRα bound in gel shift assays [156]. In addition, LXRα appeared to activate both human *CYP1A1* and *CYP1A2* simultaneously [157]. The human *CYP1A1* and *CYP1A2* genes are organized in a head-to-head orientation on chromosome 15 by sharing a common ~23-kb promoter region. Araki et al. [157] have demonstrated that two ER8-type motifs that overlap with the DR4-tye motif act as LXRα-responsive elements for the transcription of both *CYP1A1* and *CYP1A2* genes.

3. With VDR

Vitamin D receptor (VDR, NR1I1) is a comember with CAR and PXR of the NR1I subfamily and is highly expressed in intestines. In the liver, nonparenchymal cells but not hepatocytes express VDR. VDR is critical for bile acid metabolism in the intestine. Lithocholic acid (LCA), a secondary bile acid, activated the expression of VDR target genes in gastrointestinal tissues. In response to drugs that activated CAR or PXR, they cross talked with VDR to regulate *CYP3A* and *CYP24A1* genes.

CYP3A genes.Vitamin D3 (VD3) treatment induced CYP3A in Caco-2 cells [158, 159] and activated human *CYP3A4* or rat *CYP3A23* promoter in human intestine-derived LS180 cells [160]. siRNA knock-down of VDR attenuated LCA-enhanced activation of the *CYP3A4* reporter as well as VDR binding to the promoter in LS174T cells [161]. Oral LCA administration (100 mg/kg/day for 3 days) increased CYP3A protein levels in the intestines but not livers of mice [161]. Adenoviral expression of VDR did not confer mice with LCA-induced CYP3A expression in their livers [161]. Chronic treatment with drugs that activate PXR or CAR can cause metabolic bone disease in patients [162, 163]. Since CYP3A4 metabolizes active D_3 to inactive it, prolonged activation of the *CYP3A4* gene has been implicated for a cause of this side effect [164].

CYP24A gene. Vitamin D3 binds to VDR on the vitamin D3 response element (VDRE), replacing a corepressor with a coactivator for the *CYP24A1* promoter. This is a feedback mechanism against an adverse increase of active VD_3. PXR was suggested to bind to VDRE and activate it and the *CYP24A1* gene, thereby becoming a risk factor for metabolic bone diseases caused by chronic treatment with rifampicin [165]. However, this finding was challenged by another study which claimed that PXR neither binds to, nor activates, the *CYP24A1* promoter

[166]. A third study concluded that PXR binds VDRE, but this binding is negligible compared to VDR-VDRE binding in gel shift assays, and furthermore that PXR by itself does not activate VDRE [167]. However, rifampicin treatment repressed activation of the *CYP24A1* gene by active D_3. As to the mechanism of this repression, rifampicin-activated PXR binds to a VDR/corepressor SMRT/VDRE on the promoter, thereby locking the SMRT onto the promoter and not allowing it to be activated. Similarly, CAR also locked SMRT and repressed the *CYP24A1* gene. Thus, in response to their activating drugs, both PXR and CAR cross talk with VDR to repress the *CYP24A1* gene. In addition to CYP3A4, the Na/Pi co-transporter but not the *CYP24A1* gene may be the target of drugs that cause metabolic bone diseases [168].

10.6 Perspectives

The P450 enzymes within subfamilies 1, 2, and 3 are known by their roles in drug metabolism. Further research to define the molecular mechanisms of nuclear receptor-mediated induction should be continued to fully understand human susceptibility and prevention of drug treatment or environmental exposures. However, the physiological roles of these P450 enzymes have only recently come to light, such as that of CYP3A4 in vitamin D_3 metabolism [169]. In addition, CYP3A KO mice were utilized to demonstrate that CYP3A also exerts physiological roles in regulating levels of cholesterol and bile acids in vivo [170]. Since CYP3A converts cholesterol into its metabolites, the lack of CYP3A results in abnormal cholesterol metabolism which feeds back to induce related P450 enzymes, thereby increasing bile acid levels. Thus, the physiological functions of nuclear receptor-regulated so-called drug-metabolizing P450s, in particular CYP2B, should be further defined to advance this research field. AhR is also involved in the normal development and homeostasis of multiple physiological processes. To this end, endogenous ligands and/or cellular stimuli that activate nuclear receptors to regulate various physiologies must be identified.

Tremendous advances in our understanding of the regulatory mechanism no longer allow us to simply view nuclear receptors as ligand-activated transcription factors that bind to their response DNA sequences within a gene for activation. More than expected 10-years ago, cell signaling is critically involved in nuclear receptor-mediated regulation. Both CAR and PXR can be activated by cell signaling in the absence of ligands. Moreover, cell signaling may be their primary regulator and may not enable ligands to override the regulation to activate nuclear receptors. On the other hand, cell signaling confers nuclear receptors with their functional specificity as well as diversity, by regulating them at various steps such as intracellular localization and degradation, chromatin-based mechanism, selective recruitment of co-regulators and epigenetic modifications. In addition, multiple nuclear receptors co-regulate a given *CYP* gene. Future investigations must define the molecular mechanisms that regulate each of these steps, which warrant the identification of cell signaling molecules that cross talk with drugs and xenobiotics. P450 induction research should lead to new directions and to comprehend the biological functions of P450s and the roles of nuclear receptors in regulating their functions, thereby providing us with mechanistic insights into understanding human susceptibility and prevention to drug treatments and environmental exposures.

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Hormonal Regulation of Liver Cytochrome P450 Enzymes

11

David J. Waxman and Thomas K. H. Chang

Abbreviations

CIS	cytokine-inducible SH2-containing protein
CYP	cytochrome P450
GH	growth hormone
GHR	GH receptor
HNF	hepatocyte nuclear factor
3MC	3-methylcholanthrene
MSG	monosodium glutamate
SOCS	suppressor of cytokine signaling protein
STAT	signal transducer and activator of transcription.

11.1 Introduction

Interindividual differences in response to drugs are well documented [1–3]. Various factors, including sex [4–7], contribute to the variability in drug response. As first reported in the 1930s, female rats respond to a lower dosage of amobarbital [8] and experience a longer duration of action of this barbiturate [9] than male rats. In the 1960s and 1970s, sex differences in hepatic drug metabolism were identified using liver microsomes assayed in vitro using prototypic phase I cytochrome P450 (CYP) drug substrates, such as ethylmorphine, benzo[*a*]pyrene, and hexobarbital (Fig. 11.1) [10–13]. These studies showed that the sex dependence of hepatic P450 metabolism is most striking in the rat, where sex differences in metabolic rates can be fivefold or more with some drug substrates, even though the total liver P450 content is only ~20 % higher in males compared to females (Fig. 11.1). Research carried out in the 1980s resolved this discrepancy with the discovery that a subset of the multiple drug-metabolizing P450 enzymes in rat liver [14, 15] is expressed in a highly sex-dependent manner [16].

Many P450 enzymes in the *CYP* gene superfamily are active in foreign compound metabolism, in particular, genes in families *CYP1, CYP2,* and *CYP3*. These three families encompass 23 *CYP* genes (human), 50 *CYP* genes (rat), and 61 *CYP* genes (mouse) [17], and collectively carry out essentially all of the phase I CYP metabolic reactions in mammalian liver. A subset of these hepatic P450s is expressed in a sex-dependent manner subject to endocrine control [18]. The sex dependence of liver P450 enzyme expression has been widely studied at the gene (RNA) level in the rat and mouse models, but has also been reported for other species, including humans. Human liver P450 metabolism is associated with significant male–female differences in the elimination pharmacokinetics of many drugs [4, 7,

D. J. Waxman (✉)
Division of Cell and Molecular Biology, Department of Biology, Boston University, 5 Cummington Street, 02215 Boston, MA, USA
e-mail: djw@bu.edu

T. K. H. Chang
Faculty of Pharmaceutical Sciences, The University of British Columbia, 2405 Westbrook Mall, V6T 1Z3 Vancouver, BC, Canada
e-mail: thomas.chang@ubc.ca

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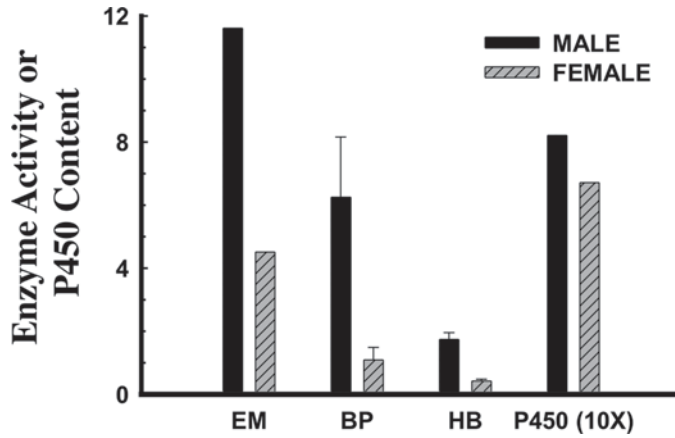


Fig. 11.1 Sex differences in rat hepatic microsomal drug metabolism. Data shown are based on enzyme assays in rat liver microsomes using the three indicated xenobiotic substrates: ethylmorphine (*EM*) [12], benzo[*a*]pyrene (*BP*) [13], and hexobarbital (*HB*) [10]. Ethylmorphine *N*-demethylase and benzo[*a*]pyrene hydroxylase activities are expressed as nanomolar product formed per minute per milligram of microsomal protein, whereas hexobarbital hydroxylase activity is expressed as nanomolar product formed per 30 min per gram of liver. Also shown is the hepatic microsomal total cytochrome P450 content, which is expressed as nanomoles per milligram of microsomal protein (values multiplied by 10) [12]. The data are shown as mean ± SD for four or five rats, except for ethylmorphine *N*-demethylase and total P450 which are based on a pool of six livers

19], and is in part determined by age, sex, and hormone status [4, 5, 7, 19–21]. Overall, more than 1000 genes show significant sex differences in expression in human liver, as indicated by global microarray analysis [22]. The sex-differentially expressed human genes affect diverse physiological functions, including metabolic processes that impact lipid profiles associated with sex differential risk of human coronary artery disease [22]. More than 400 of the sex-dependent genes in human liver have mouse orthologs that show sex-biased hepatic expression regulated by the polypeptide hormone growth hormone (GH; see Sect. 11.4.2.2), suggesting GH plays a similar regulatory role in the human liver [22]. Studies of the mechanisms by which GH and other endocrine factors regulate rat and mouse liver P450 enzymes may therefore help elucidate corresponding regulatory processes in human liver, which can impact P450-catalyzed reactions affecting the metabolism of lipids, endogenous steroids, drugs, and environmental chemicals.

This chapter reviews the sex-dependent hepatic P450s; their regulation by endocrine factors; and the underlying molecular, genomic, and epigenetic mechanisms of action governing this regulation. We also discuss the role of hepatic P450s in steroid hormone metabolism, as well as the environmental and pathophysiologic factors that can perturb hormonal status and thereby impact the sex-dependent expression of hepatic P450s. Lastly, we discuss the effects of sex steroid hormones on hepatic expression of xenobiotic-inducible liver P450 enzymes and the role of specific receptors in regulating sex steroid induction of these P450s.

11.2 Sex-Dependent Liver P450 Enzymes

The physiological requirements with respect to steroid hormone hydroxylation differ between the sexes, and not surprisingly, several steroid hydroxylase liver P450s are expressed in a sex-dependent manner [16, 23]. Rat P450 enzymes CYP2C11 and CYP2C12 are prototypic examples of sex-specific steroid hydroxylase liver P450 enzymes (Table 11.1), and they have been a major focus of studies of the underlying endocrine factors, as well as the cellular and molecular regulatory mechanisms that govern

Table 11.1 Hormonal regulation of sex-dependent rat liver P450 enzymes

		Hormonal regulation[c]		
		Testosterone hydroxylase	Androgenic	
CYP enzyme[a]		Activities[b]	Imprinting[d]	Thyroid hormone[e]
I. Male-specific				
	2A2	15α	++	+/–
	2C11	2α, 16α	++	+/–
	2C13	6β[f], 15α	++	ND
	3A2	6β, 2β	++	––
	3A18	16β, 2β, 15β, 16α	ND	ND
	4A2	(see footnote g)	ND	–
II. Female-specific				
	2C12	15β[h]	––	+/–
III. Female-predominant[i]				
	2A1	7α	ND	–
	2C7	16α	ND	++
	3A9	6β	ND	ND
	5α-reductase	–	––	++

[a] P450 gene designations are based on the systematic nomenclature of [322]. The table is modified from [102]
[b] The major sites of testosterone hydroxylation catalyzed by the individual P450 proteins are shown. Testosterone metabolites specific to the P450's activity in rat liver microsomal incubations are underlined. Based on [15, 16, 26, 38, 45] and references therein
[c] "++" indicates a positive effect on adult enzyme expression, while "—" indicates a suppressive effect. "–" indicates a lesser degree of suppression, while "+/–" indicates no major effect. ND—not determined in a definitive manner
[d] For further details, see [35, 37, 65]
[e] Based on [48, 110, 122, 183, 184]
[f] Purified CYP2C13 exhibits high testosterone hydroxylase activity in a purified enzyme system, but this enzyme makes only marginal contributions to liver microsomal testosterone hydroxylation [323]
[g] CYP4A2 catalyzes fatty acid ω-hydroxylation, but it does not catalyze testosterone hydroxylation
[h] 15β-hydroxylation of steroid sulfates [31]. CYP2C12 also catalyzes weak testosterone 15α- and 1α-hydroxylase activities
[i] Liver expression of these enzymes is readily detectable in both male and female rats, but at a three- to tenfold greater level in females as compared to males

sex-specific liver gene expression. CYP2C11 is the major male-specific testosterone 16α- and 2α-hydroxylase in adult rat liver, and is induced at puberty in males but not females [24, 25] under the influence of neonatal androgenic imprinting (programming) [26]. By contrast, the steroid sulfate 15β-hydroxylase CYP2C12 is expressed in a female-specific manner in adult rat liver [26, 27]. Other sex-dependent rat liver P450 enzymes include the male-specific enzymes CYP2A2, CYP2C13, CYP3A2, CYP3A18, and CYP4A2, and the female-predominant enzymes CYP2A1, CYP2C7, and CYP3A9 (Table 11.1).

11.2.1 Steroid Hormones as Substrates for Sex-Dependent Liver P450s

The precise physiological functions of the endocrine-regulated liver P450s are not known; however, the finding that steroid hormones are metabolized by liver P450 enzymes with a much higher degree of regiospecificity and stereoselectivity than many foreign compound substrates [16] suggests that these endogenous lipophiles serve as physiological P450 substrates. Testosterone is hydroxylated in a regiospecific and stereoselective manner by multiple sex-dependent rat liver P450 enzymes (Table 11.1). Liver microsomal testosterone hydroxylation at the 7α-, 15α-, 2α-, and 6β-positions is respectively catalyzed

by CYP2A1, CYP2A2, CYP2C11, and CYP3A enzymes [26, 28–30]. In contrast, CYP2C12 catalyzes the 15β-hydroxylation of steroid sulfates [31]. Testosterone 7α-hydroxylation, testosterone 15α-hydroxylation, testosterone 2α-hydroxylation, testosterone 6β-hydroxylation, and steroid sulfate 15β-hydroxylation can be used as specific catalytic markers for rat liver microsomal enzymes CYP2A1, CYP2A2, CYP2C11, CYP3A, and CYP2C12, respectively [15, 16]. Other steroid hormones, including androstenedione and progesterone, also undergo stereoselective and regiospecific hydroxylation catalyzed by rat [26, 32] and human [33] liver P450 enzymes.

11.3 Developmental Regulation of Sex-Dependent Rat Liver P450s

Many of the sex-dependent liver CYP enzymes are subject to complex developmental regulation and endocrine control (Table 11.1). Rat CYP2C11, the major male-specific androgen 2α- and 16α-hydroxylase of adult liver, is not expressed in immature rats but is induced dramatically at puberty (beginning at 4–5 weeks of age) in male but not female rat liver [24, 25]. Three other male-specific rat liver cytochromes P450 exhibit a similar developmental profile: CYP2A2 [34, 35], CYP2C13 [36, 37], and CYP3A18 [38, 39]. In contrast, another adult male-specific liver P450, CYP3A2, is expressed in prepubertal rat liver at similar levels in both sexes, but is selectively repressed at puberty in female liver [26, 29, 40, 41]. CYP2C12 is expressed at a moderate level in both male and female rats at 3–4 weeks of age. Beginning at puberty (~30–35 days postnatal), CYP2C12 levels are further increased in females while they are fully repressed in males [26, 27]. Several other female-predominant liver enzymes are increased in expression at puberty in adult female rats. These include: CYP2C7 [36, 42], which catalyzes retinoic acid 4-hydroxylation [43]; CYP3A9 [44], which catalyzes steroid 6β-hydroxylation [45]; and steroid 5α-reductase, which is not a CYP enzyme but plays an important role in steroid metabolism in adult female rats [26, 46]. Finally, CYP2A1, a female-predominant steroid 7α-hydroxylase that is expressed in both sexes shortly after birth, is repressed at puberty to a greater extent in male than in female rat liver [26, 47, 48]. Each of these sex-dependent P450 enzymes is expressed primarily in the liver, although low-level expression in one or more extrahepatic tissues may occur in some cases [49–51].

The changes in liver P450 levels during postnatal development have been studied in both rat and mouse liver at the RNA level using genome-wide expression microarrays. In rat liver, sex differences in expression are seen as early as 2 weeks postnatally for a few genes; however, widespread sex differences do not appear until the onset of puberty (~5 weeks of age) [52]. Analysis of the developmental changes in gene expression in mouse liver has shown that many female-biased genes are downregulated in male liver at puberty, while male-biased genes are upregulated. Many fewer developmental changes affecting sex-biased genes occur in female liver [53]. In both male and female mouse liver, genes upregulated from 3 to 8 weeks of age were enriched for genes positively regulated by the transcription factor hepatocyte nuclear factor 4α (HNF4α), which is known to play a critical role in liver development and differentiation [54, 55], while genes downregulated during the same developmental period were enriched for genes negatively regulated by HNF4α [53, 56]. Several female-biased transcriptional regulators, encoded by *Cux2, Trim24,* and *Tox* [57], displayed sex-differential expression at 4 weeks of age, i.e., just prior to the emergence of extensive sex differences in liver gene expression. One or more of these transcription factors could contribute to the sex-biased developmental changes in P450s and that of many other liver-expressed genes that emerge at puberty [53]. Detailed studies of one of these factors, Cux2, support this conclusion [58] (see Sect. 11.4.2.4.3).

During senescence, there is a general loss of sex-dependent enzyme expression; this largely reflects a decrease in male P450 levels and an increase in expression of female-biased P450s, as seen in livers of aging male rats [59–63]. These changes appear to be related to the age-dependent

reduction in the secretion of GH-releasing factor and associated changes in the sex-dependent pattern of pituitary GH secretion [64], which is a major regulator of the sex-dependent expression of liver CYP enzymes (see Sect. 11.4.2). In aging male rats, the decline in hepatic CYP2C11 expression is not accompanied by a decrease in the GH-activated transcription factor signal transducer and activator of transcription 5b (STAT5b) [62], whereas the increase in hepatic CYP2C12 expression is accompanied by an increase in HNF3β [62], which is also involved in the regulation of sex-dependent liver P450 enzymes (see Sect. 11.4.2).

11.4 Hormonal Control of Liver P450 Expression

11.4.1 Regulation by Gonadal Hormones

Gonadal steroids play an important role in regulating the sex-dependent pattern of hepatic steroid and drug metabolism and P450 expression. However, gonadal steroids largely act indirectly via their effects on the hypothalamus, which regulates the pituitary gland and determines its sex-dependent temporal pattern of GH secretion (see Sect. 11.4.2).

11.4.1.1 Testosterone

11.4.1.1.1 Distinct Effects of Neonatal Androgen and Adult Androgen

Gonadal hormones play an essential role in determining the expression of the major sex-specific rat liver P450 forms at adulthood. For testosterone, there are two distinct periods of postnatal hormone production, neonatal and postpubertal, and each period makes a distinct contribution to the expression of the sex-dependent liver P450s at adulthood. Castration of male rats at birth eliminates both periods of testosterone production and thereby abolishes normal adult male liver expression of the male-specific P450 enzymes CYP2A2 [35], CYP2C11 [25, 26, 36, 65, 66], CYP2C13 [37], and CYP3A2 [26, 65, 66]. Adult male liver expression of CYP2C13 RNA [37] is also abolished in birth-castrated rats, indicating that enzyme expression is regulated at a pretranslational step. Treatment of birth-castrated male rats with testosterone during the neonatal period partially restores expression of these male-specific P450s at adulthood [26, 35, 37]. A brief period of neonatal androgen exposure is thus sufficient to "imprint" or irreversibly program the male rat to express these P450 enzymes later in adult life. These effects of neonatal androgen on male-specific P450 enzymes are very similar to the androgenic imprinting effects described in earlier studies of liver microsomal steroid hydroxylase activities [46, 67, 68], several of which can be associated with specific liver P450 enzymes [16].

Administration of testosterone to birth-castrated male rats during the neonatal period (typically during the first few days of life) partially restores normal adult male expression levels of CYP2C11 [26, 66] and CYP2C13 [37] at adulthood, indicating that neonatal androgen alone is insufficient for full adult expression of these male-specific P450s. In contrast, the combination of neonatal androgen treatment with adult androgen exposure fully restores normal adult male levels of the male-specific P450s [26]. Testosterone treatment of adult male rats that were castrated either neonatally or prepubertally substantially increases the expression of CYP2C11 [65, 66, 69, 70] and CYP2C13 [37]. However, in contrast to the irreversible imprinting effects of neonatal androgen treatment, the effects of adult androgen exposure are likely to be reversible; this is indicated by the partial loss of CYP2C11 in male rats castrated at adulthood [25, 26] and by the reversal of this loss by the synthetic androgen methyltrienolone [71]. Similarly, the continued presence of testosterone at adulthood is required to maintain normal adult expression of CYP3A2, since castration at 90 days of age reduces hepatic CYP3A2 messenger RNA (mRNA) levels by >80%, but this can be restored by subsequent administration of testosterone to the adult rat [72]. Thus, while neonatal testosterone imprints the rat for expression of male-specific P450 enzymes beginning at puberty, when the demand for P450-dependent liver steroid metabolism increases, the additional

presence of androgen during the pubertal and postpubertal periods is required to maintain full enzyme expression during adult life [73, 74].

11.4.1.1.2 Testosterone Suppression of Female Enzymes

Testosterone suppresses expression of the female-specific CYP2C12 as well as the female-predominant enzymes CYP2A1 and steroid 5α-reductase. Hepatic CYP2C12 content is reduced in intact, adult female rats exposed chronically to testosterone [65] or to the synthetic androgen methyltrienolone [27]. Similarly, treatment of neonatally or prepubertally ovariectomized rats with testosterone, either neonatally or pubertally, results in a major decrease in liver microsomal steroid 5α-reductase activity [65, 73]. Birth castration of male rats increases the adult levels of hepatic CYP2A1, but testosterone administration to these animals re-masculinizes (i.e., decreases) the levels of this P450 [75]. Androgens thus exert a suppressive effect on liver CYP2A1 expression. Studies of the effect of testosterone on the expression of the female-predominant CYP2C7 are inconclusive [69, 76].

11.4.1.1.3 Mechanisms of Testosterone Regulation

Testosterone's primary effects on liver P450 profiles are mediated by the hypothalamic–pituitary axis [77] and its control of the sex-dependent pattern of pituitary GH secretion [78, 79]. Consistent with this conclusion, testosterone has only minor effects on liver enzyme profiles in hypophysectomized rats in most [80] but not all [81, 82] instances. Rather, as discussed in Sect. 11.4.2, the effects of testosterone on liver P450 expression are thought to be mostly indirect, being mediated by sex differences in pituitary GH secretory patterns.

11.4.1.2 Estrogen

A role for estrogen in the expression of the female-specific liver P450 enzymes is suggested by the effects of ovariectomy at birth, which reduces, but does not abolish, hepatic expression of CYP2C7, CYP2C12, and steroid 5α-reductase in adult female rats [26, 65, 69], and by the restoration of normal adult enzyme levels by estrogen replacement. Ovariectomy during adulthood [83] or neonatal administration of an estrogen receptor antagonist, tamoxifen [84], reduces hepatic CYP3A9 levels in adult female rats. The decrease in CYP3A9 expression in ovariectomized rat liver can be reversed by estrogen treatment [83]. By contrast, estradiol suppresses hepatic CYP2C11 in both intact and castrated male rat liver [65, 69]. However, the absence of CYP2C11 in adult female rat liver is not due to a direct negative effect of estrogen. Thus, ovariectomy alone does not induce CYP2C11 expression in female rat liver [26, 65, 69]. In male rats, the suppression of CYP2C11 by estradiol may be irreversible, as demonstrated by the major loss of this P450 in livers of adult male rats exposed to estradiol during the neonatal period or at puberty. However, this effect is not a consequence of a direct action of estradiol on the liver, since estradiol does not impact hepatic CYP2C11 levels in hypophysectomized rat liver [81]. Rather, the effects of estradiol on hepatic P450 expression involve the hypothalamic–pituitary axis, and most likely result from an estrogen-dependent increase in the interpeak baseline levels of plasma GH [77, 85]. This effect of estradiol may be sufficient to alter the sex-specific effects of pituitary GH secretion since, as discussed in greater detail below, recognition of a "masculine" GH pulse by hepatocytes requires an obligatory recovery period during which there is no plasma GH and hence no stimulation of hepatocyte GH receptors (GHRs) [86]. In addition, estrogen may antagonize the induction of CYP2C11 by testosterone as suggested by the absence of androgen imprinting of this P450 in intact female rats treated with neonatal or pubertal testosterone [69, 70]. Indeed, the stimulatory effect of testosterone on the male, pulsatile pattern of pituitary GH secretion can be blocked by the presence of intact ovaries in female rats [79]. Interestingly, prepubertal treatment of intact (i.e., nonovariectomized) female rats with tamoxifen enhances the induction of CYP2C11 and CYP3A2 expression by pubertal and postpubertal androgen [87]. The neuroendocrine mechanisms responsible for the antagonistic effects

of estrogen on androgen imprinting remain to be elucidated.

Exposure to xenoestrogens during the neonatal and adult periods can influence the effects of GH on sex-specific liver P450 levels. Administration of bisphenol A to female rats on postnatal days 1–10 decreases hepatic *CYP2C12* gene expression and has no effect on *CYP2C11* gene expression, when assessed postpubertally at 5 months of age [88]. The decrease in CYP2C12 is associated with an increase in pituitary GH content. By comparison, treatment of adult male rats with bisphenol A suppresses hepatic microsomal CYP2C11 and CYP3A2 protein and enzyme activities [89]. The mechanistic basis for the neonatal and adult exposure effects of bisphenol A on hepatic CYP2C11 and CYP2C12 expression have not been identified.

11.4.2 Regulation by GH

11.4.2.1 Sex-Dependent GH Secretory Profiles

In many mammalian species, the pituitary gland secretes GH into the bloodstream in a highly regulated temporal fashion, which differs between males and females. This sex-dependent secretion of GH is most striking in rats and mice [90–93], but key features are conserved in humans [94–98] (Fig. 11.2). In the adult male rat, GH is secreted by the pituitary gland in an intermittent (i.e., pulsatile) manner characterized by high peaks of hormone in plasma (200–300 ng/ml) each 3.5–4 h followed by a period of very low or undetectable circulating GH (< 1–2 ng/ml). By contrast, in the adult female rat, GH is secreted more frequently (multiple pituitary se-

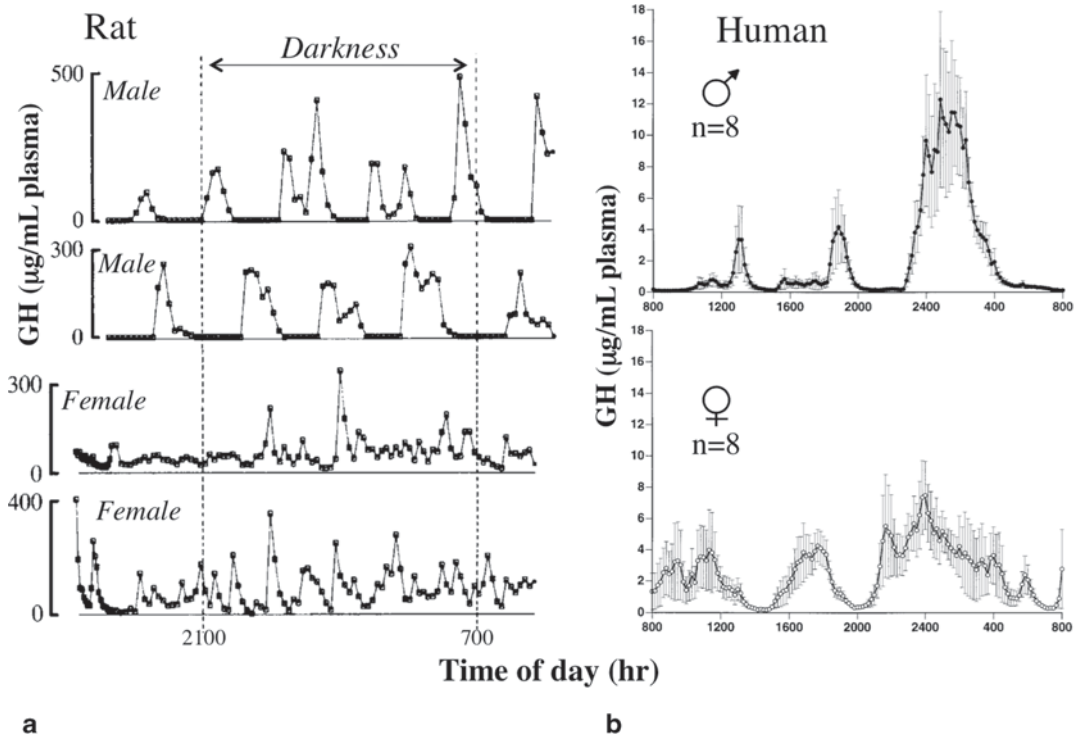


Fig. 11.2 Sex differences in plasma growth hormone (*GH*) profiles in adult rats (**a**) and humans (**b**). Shown are plasma GH profiles measured during the course of a single day in each of two individual male and two individual female rats (panel **a**) and mean plasma GH profiles assayed in $n = 8$ individual men and $n = 8$ individual women (panel **b**). Data shown are from [330] (panel **a**) and [331] (panel **b**)

cretory events per hour) and in a manner such that the plasma GH pulses overlap and the hormone is present in circulation at significant levels at nearly all times [80] (Fig. 11.2a). Human males also show well-defined plasma GH-free periods between major secretory periods, whereas in human females, the GH-free periods are of limited duration (Fig. 11.2b). Hypophysectomy and GH replacement experiments demonstrate that these sex-dependent plasma GH profiles are, in turn, responsible for establishing and for maintaining the sex-dependent patterns of liver P450 gene expression in rats [25, 27, 35, 86, 99] and mice [100, 101] (for earlier reviews, see [102, 103]). Clinical studies in humans also demonstrate a role for GH [104–108] and its sex-dependent plasma secretory patterns [109] in regulating P450-dependent drug metabolism.

Studies in the rat model reveal three distinct responses of liver P450s to plasma GH profiles (Fig. 11.3):

1. Continuous plasma GH, a characteristic of adult female rats, stimulates hepatic expression of female specific enzymes, such as CYP2C12 and steroid 5α-reductase [27, 75], and female-dominant liver enzymes, such as CYP2A1, CYP2C7, and CYP3A9 [75, 110–112]. Hepatic levels of CYP2C12 and steroid 5α-reductase are undetectable in hypophysectomized female rats, but can be restored to near-normal female level by continuous GH

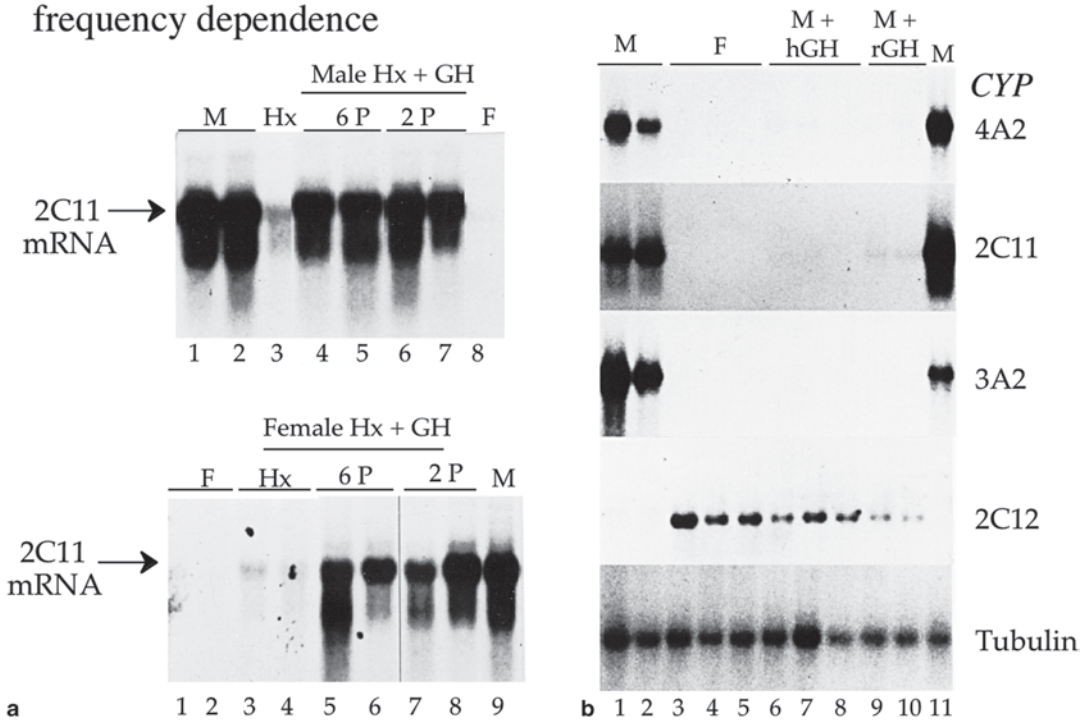
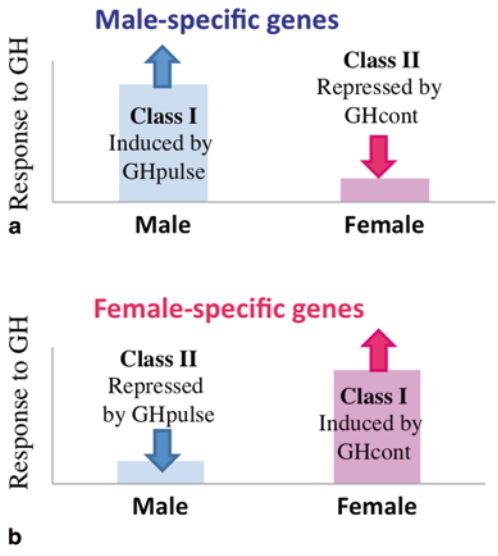


Fig. 11.3 Impact of plasma growth hormone (*GH*) profile on sex-dependent rat hepatic cytochrome P450 (*CYP*) mRNA levels. Shown are Northern blots probed with oligonucleotide probes specific for each of the indicated CYP RNAs. Panel **a** shows the male (*M*)-specific expression of *CYP2C11,* which is not expressed in hypophysectomized rat liver (*Hx*) and is induced in livers of both male and female (*F*) hypophysectomized rats given either two or six pulses (*P*) of GH/day for 7 days. Data based on [86]. Panel **b** shows the effects of continuous rat (r) or human (h) GH infusion in male rats (lanes 6–10) on the mRNA levels of *CYP4A2, CYP2C11,* and *CYP3A2* (all male-specific; lanes 1, 2, 11 vs. lanes 3–5), as well as *CYP2C12,* which is induced. Tubulin RNA is shown as a loading control. The figure is based on [18]

infusion [75, 113, 114, 115]. This restoration can be achieved with as little as 12–25% of the physiological levels of GH [115]. Greater levels of GH are required to induce expression of CYP2C12 and steroid 5α-reductase in hypophysectomized male rats [116].

2. Intermittent plasma GH pulses, which are characteristic of adult male rats, induce expression of the male-specific liver enzyme CYP2C11 (Fig. 11.3a) and its associated testosterone 2α-hydroxylase activity [25, 73, 86, 99]. The stimulatory effects of intermittent GH stimulation on this "class I" male P450 enzyme can be distinguished from the effects of GH pulses on a second group of male-specific liver P450s ("class II" enzymes CYP2A2, CYP2C13, CYP3A2, CYP3A18, and CYP4A2). In contrast to the class I CYP2C11, class II male-specific P450s are not obligatorily dependent on GH pulses, as judged by their high level of expression in the absence of GH, as demonstrated in hypophysectomized rats of both sexes [35, 37, 39, 115–119] (Fig. 11.4). Nevertheless, liver expression of the class II enzymes CYP2A2 and CYP3A2 is induced when intermittent GH pulses are given to adult male rats that are depleted of circulating GH by neonatal monosodium glutamate (MSG) treatment [118].
3. Continuous GH exposure exerts major negative regulatory effects on male liver P450 enzyme expression, as revealed by the marked suppression of each of the class I and class II male-specific rat liver P450s following continuous GH treatment of intact male rats (Fig. 11.3b). In some cases, this effect can be achieved at low circulating GH levels, corresponding to only 3–12% of the physiological



Male Class	Male hypox	Female hypox	Rat Genes	Mouse Genes
I	Down	No change	*Cyp2c11* *Mup4*	*Gstp1* *Cyp7b1* *Cyp4a12*
II	No change	Up	*Cyp2a2* *Cyp2c13* *Cyp4a2*	*Nox4* *Bcl6* *Gsta1*

Female Class	Male hypox	Female hypox	Rat Genes	Mouse Genes
I	No change	Down	*Cyp2c12* *A1bg* *Cux2*	*Cyp3a41* *Fmo3* *Cux2*
II	Up	No change	*Adh3* *Adh4*	*Cyp2b9* *Cyp4a10* *Tox*

Fig. 11.4 Class I and class II sex-specific genes. Class I male-specific genes are induced by plasma growth hormone (*GH*) pulses in male liver (**a**) and class I female-specific genes are induced by the more continuous female plasma GH profile in female liver (**b**). Class II male-specific genes are repressed in female liver by the female plasma GH profile (**a**) and class II female-specific genes are repressed in male liver by the male plasma GH profile (**b**). Consequently, the loss of GH following hypophysectomy ("hypox") leads to downregulation of class I male-specific genes and to upregulation of class II female-specific genes (derepression) in male liver. Hypophysectomy also leads to downregulation of class I female-specific genes and to upregulation of class II male-specific genes (derepression) in female liver (table at *right*). Class II male-specific genes do not require male plasma GH pulses for expression, and therefore are most often unchanged in expression in male liver following hypophysectomy, and, correspondingly, class II female-specific genes do not require the female pattern of GH stimulation for expression and are most often unchanged in expression in male liver following hypophysectomy. Specific examples of each gene class are shown in the last column

GH level in adult female rats [116]. The high-level expression of class II P450 mRNAs seen in the absence of GH pulses, i.e., in hypophysectomized male rats, is also suppressed by continuous GH treatment, indicating that continuous GH actively suppresses P450 gene expression, and does not simply act by abolishing the pulsatile plasma GH pattern. GH suppression is also a key determinant of the lower responsiveness of female rats to phenobarbital induction of CYP2B1 [120, 121], and probably also the lower responsiveness of female liver to the induction of CYP4A enzymes by peroxisome proliferators such as clofibrate [122].

The response of the class II male P450 genes to hypophysectomy of female rats, which derepresses (i.e., increases) female liver P450 enzyme levels to near-normal intact male liver enzyme levels, demonstrates that the class II male liver P450s are subject to negative pituitary regulation in female rat liver, where their expression is strongly repressed by the near-continuous pattern of plasma GH exposure (Fig. 11.4). These patterns of hormonal regulation are summarized in Table 11.2, which presents the responses of prototypic sex-specific liver P450s to continuous and intermittent GH treatment applied to intact, hypophysectomized, and neonatal MSG-treated rats. Importantly, these patterns of response to pituitary GH ablation by hypophysectomy are recapitulated when the effects on sex-specific gene expression are examined on a global scale by microarray analysis, as seen in both rat liver [123] and mouse liver [124]. Interestingly, the latter studies revealed that male liver displays an intrinsically greater responsiveness than female liver to the rapid effects of a pulse of GH. Thus, many individual male-specific genes are induced rapidly (within 30 min) in livers of hypophysectomized male but not hypophysectomized female mice treated with a single plasma pulse of GH [124]. Thus, GH pulse responsiveness is in part determined by *intrinsic* sex-specific factors, which may result from prior hormone exposure (epigenetic mechanisms) or genetic factors that are pituitary independent and could contribute to sex differences in the predisposition to liver cancer or other hepatic pathophysiologies [125].

11.4.2.2 Transcriptional Effects of GH on CYP Genes

GH regulates steady-state liver P450 mRNA levels in parallel with P450 protein and P450 enzyme activity levels, all but ruling out major regulation by translational and posttranslational

Table 11.2 Response of sex-specific rat CYPs to GH

	Intact rats			Hypophysectomized rats			MSG-treated rats		
CYP	F	M	M	F	M	M	M	M	M
			+			+	+		+
			GH_{cont}			GH_{int}	GH_{cont}		GH_{int}
CYP2C11[a] (Male class I)	–	++	–	–	–	++	–	–	++
CYP2A2[b] (Male class II)	–	++	–	++	++	++	+/–	–	++
CYPC12[c] (Female specific)	++	–	++	–	–	–	++	–	–

CYP cytochrome P450, *F* female, GH_{cont} continuous growth hormone, GH_{int} intermittent (pulsatile) growth hormone, *M* male

"++" indicates a positive effect, "–" indicates a suppressive effect, and "+/–" indicates no major effect

[a] Data are based on [25, 41, 86, 115, 116, 118, 119, 135, 324–328]

[b] Data are based on [35, 115, 116, 118, 119, 135, 327–329]

[c] Data are based on [113, 115, 116, 135, 325, 327–329]

mechanisms, such as regulation of P450 protein turnover. Induction of CYP2C12 mRNA by continuous GH requires ongoing protein synthesis [126], suggesting either an indirect induction mechanism or a requirement for one or more protein components that may have a short half-life. Analysis of liver nuclear RNA demonstrates that unprocessed, nuclear CYP2C11 and CYP2C12 RNA respond to circulating GH profiles in a manner that is indistinguishable from the corresponding mature, cytoplasmic mRNAs [122]. Consequently, RNA splicing, transport of CYP2C11 and CYP2C12 mRNA to the cytoplasm, and cytoplasmic P450 mRNA stability are unlikely to be important GH-regulated control points for sex-specific P450 expression. Moreover, nuclear run-on transcription analyses have established that GH regulates the sex-specific expression of the CYP2C11 and CYP2C12 genes at the level of transcript initiation [122, 127]. Transcription is also the major step for regulation of the male class II CYP2A2 and CYP2C13 mRNAs [122, 127], whose male-specific expression is primarily a consequence of the suppressive effects of continuous GH exposure in adult female rats [35]. Thus, transcription initiation is the key step at which the three distinct effects of GH outlined in Sect. 11.4.2.1 are operative: stimulation of CYP2C11 expression by pulsatile GH, suppression of both class I and class II male-specific P450s by continuous GH, and stimulation of CYP2C12 expression by continuous GH [122]. Class II male-specific rat liver genes, such as *CYP2A2* and *CYP2C13* (Sect. 11.4.2.1), are downregulated within 30 min of GH pulse treatment, as determined by heterogeneous nuclear RNA (primary transcript) analysis [123], suggesting that transcription of these genes is restricted to the GH-free interpulse period in adult male rat liver.

Consistent with the finding that GH regulates sex-dependent liver CYPs by transcriptional mechanisms, the 5′-flanking DNA segments of both the *CYP2C11* [128] and *CYP2C12* genes [129] contain specific DNA sequences that interact in a sex-dependent and GH-regulated manner with nuclear DNA-binding proteins (putative transcription factors) that are differentially expressed in male versus female rat liver [122, 130]. These DNA sequences are hypothesized to include GH response elements that contribute to the sex-specific transcription of the *CYP2C11* and *CYP2C12* genes. Two negative regulatory elements ("silencer elements") were also identified in the CYP2C11 promoter; however, their significance with respect to GH regulation and sex-specific P450 expression is as yet unclear [131]. More detailed, genome-wide studies of sex-specific mouse *CYP* genes and their regulatory elements, and their interactions with liver-enriched and GH-responsive transcription factors are discussed below (Sect. 11.4.2.4).

11.4.2.3 Cellular Mechanisms of GH Signaling

The cellular mechanisms whereby pituitary GH secretory profiles regulate expression of the sex-dependent liver P450s are only partially understood. GH can act directly on the hepatocyte to regulate liver P450 expression, as demonstrated by the responsiveness of primary rat hepatocyte cultures to continuous GH-stimulated expression of CYP2C12 mRNA; however, these effects do not involve insulin-like growth factor (IGF)-I, a mediator of several of GH's physiological effects on extrahepatic tissues [126, 132]. Discrimination by the hepatocyte between male and female plasma GH profiles is likely to occur at the cell surface, where a higher level of GHRs (see below) is found in female as compared to male rats [133]. This sex difference in cell surface GHR abundance may, at least in part, be due to differential effects of intermittent versus continuous GH stimulation of GH signaling leading to receptor internalization and/or downregulation [134] and could play a role in the activation of distinct intracellular signaling pathways by chronic (female) as compared to intermittent (male) GH stimulation.

14.4.2.3.1 Significance of GH Pulse Frequency

It is important to determine which of the three descriptive features of a GH pulse—namely, GH pulse duration, GH pulse height, and GH pulse frequency—is required for proper recognition of

a GH pulse as "masculine." Direct measurement of the actual plasma GH profiles achieved when GH is administered to hypophysectomized rats by twice-daily subcutaneous (s.c.) GH injection (i.e., the intermittent GH replacement protocol commonly used to stimulate CYP2C11 expression) has revealed broad peaks of circulating GH, which last as long as 5–6 h [86]. These sustained GH "pulses" are nonphysiological; nevertheless, they are effective in stimulating expression of the male-specific CYP2C11, provided that they are not administered in close succession. Physiological GH pulse duration (<2 h) is therefore not required to elicit a male *CYP* gene response. Studies carried out in GH-deficient rat models (either dwarf rats or rats depleted of adult circulating GH by neonatal MSG treatment) demonstrate that GH pulse height is also not a critical factor for stimulation of CYP2C11 expression [127, 135]. This finding can be understood in terms of the K_d of the GH–GHR complex, which at 10^{-10} M (~2 ng/ml) [136], is only ~1% of the peak plasma hormone level in adult male rats. In contrast, GH pulse frequency is a critical determinant for GH stimulation of a male pattern of liver P450 expression, as shown in hypophysectomized rats given physiologic replacement doses of GH for 7 days by intermittent intravenous injections at frequencies of 2, 4, 6, or 7 times/day [86]. Analysis of liver CYP2C11 RNA levels in these rats revealed a normal male pattern of liver CYP2C11 gene expression in response to six GH pulses per day (which approximates the normal male plasma GH pulse frequency), as well as in response to GH pulses given at lower frequencies, e.g., twice daily (e.g., Fig. 11.3a). However, hypophysectomized rats are not masculinized by seven daily GH pulses, indicating that the hepatocyte does not recognize the pulse as "masculine" if GH pulsation becomes too frequent. Hepatocytes thus require a minimum GH off time (~2.5 h in the hypophysectomized rat model used in these studies), which implies the need for an obligatory recovery period to effectively stimulate CYP2C11 expression. This condition is not met in the case of hepatocytes exposed to GH continuously (female hormone profile). This recovery period may serve to reset the cellular signaling apparatus, e.g., by replenishing GHRs at the cell surface (see below).

14.4.2.3.2 Role of GHR

The effects of GH on hepatocytes and other responsive cells are transduced by GHR, a 620-amino-acid cell surface transmembrane protein [136] belonging to the cytokine receptor superfamily [137]. GHR lacks intrinsic tyrosine kinase activity, but relies on its interactions with Janus kinase 2 (JAK2), a GHR-associated tyrosine kinase that is activated following GH binding to GHR (Fig. 11.5). GHR is composed of a 246-amino-acid extracellular domain that binds GH, a single transmembrane segment, and a 350-amino-acid intracellular domain that interacts with JAK2 and participates in the intracellular signaling events stimulated by GH [136, 138]. X-ray crystallographic and other studies establish that a single molecule of GH binds in a stepwise manner to a predimerized pair of GHR molecules to yield an activated receptor complex: GH + 2 GHR -> GH–$(GHR)_2$ [139, 140]. GHR is proposed to initially contact GH via amino acids comprising GH site 1, followed by interaction with site 2 on the GH molecule to give a heterotrimeric GH–$(GHR)_2$ complex. Receptor activation is thought to result from a rotation of the receptor monomers within the complex [141, 142]. These conformational changes are necessary, and probably sufficient, for stimulation of GH-induced intracellular signaling events [143].

In adult male rat liver, GHR internalizes to an intracellular compartment coincident with its stimulation by plasma GH pulses, and then reappears at the cell surface at the time of the next hormone pulse [144, 145]. GHR undergoes endocytosis constitutively, i.e., in a ligand-independent manner, but is also subject to GH-stimulated internalization [146]. GHR internalization is rapid in GH-treated liver cells [147] and is mediated by coated vesicles that ultimately take the receptor to lysosomes for degradation. GHR endocytosis and degradation require: (1) an intact ubiquitin conjugation system, which targets a specific 10-amino-acid-long cytoplasmic GHR tail sequence; (2) the ubiquitin ligases SCF(βTrCP) and CHIP [148, 149]; and (3) 26S proteasome activ-

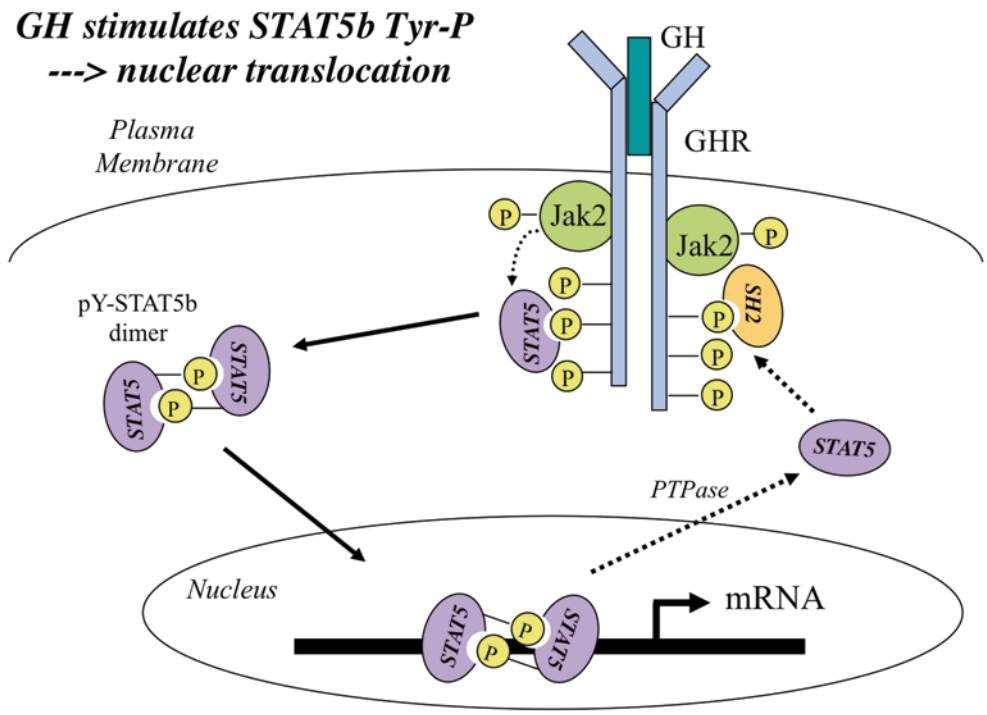


Fig. 11.5 Role of growth hormone (*GH*), GH receptor, and the tyrosine kinase Janus kinase 2 (*JAK2*) in activation of signal transducer and activator of transcription 5b (*STAT5b*) by tyrosine phosphorylation. JAK2 tyrosine phosphorylates itself and multiple tyrosine residues on the cytoplasmic tail of growth hormone receptor (*GHR*). Several of these sites serve as docking sites that recruit *STAT5b* to the GHR–JAK2 complex. STAT5b is then tyrosine-phosphorylated by JAK2, whereupon it dimerizes by mutual SH2 domain–phosphotyrosyl–STAT5b interactions, then translocates to the nucleus where it binds to DNA regulatory elements upstream of its target genes. The STAT5 activation cycle is reversed by the action of a phosphotyrosine phosphatase, which leads to recycling of inactive STAT5 monomers back to the cytoplasm. The figure is based on [332]. *mRNA* messenger RNA

ity, as evidenced by the inhibitory effects of the proteasome inhibitors MG132 and epoxomicin [147]. In liver cells, the GH-inducible suppressor of cytokine signaling protein (SOCS)/CIS family member CIS, a negative feedback regulator of GHR signaling, plays an important role in GHR internalization leading to termination of GHR signaling [147]. Although cellular ubiquitination activity is required for receptor endocytosis, GHR itself does not need to undergo ubiquitination, as shown using a mutant GHR devoid of its cytoplasmic lysine residue targets for ubiquitination [150, 151]. Thus, the ubiquitin–proteasome system is a major regulator of intracellular GHR trafficking.

11.4.2.4 Role of STAT5b in Sex-Dependent CYP Expression

11.4.2.4.1 GH Signaling Pathways Involving STAT Transcription Factors

How does GH impart sex-dependent transcriptional regulation to liver P450 genes? To answer this question, we may consider the following hypotheses: (1) the cell surface GHR can discriminate between the male and female plasma GH patterns; and (2) GH-activated GHR signals to the nucleus by two distinct intracellular signaling pathways, one in response to GH pulses and the other in response to persistent GH stimulation (Fig. 11.6). Studies of GH-induced signal transduction pathways [152–154] have highlighted the importance of the GH-bound receptor dimer

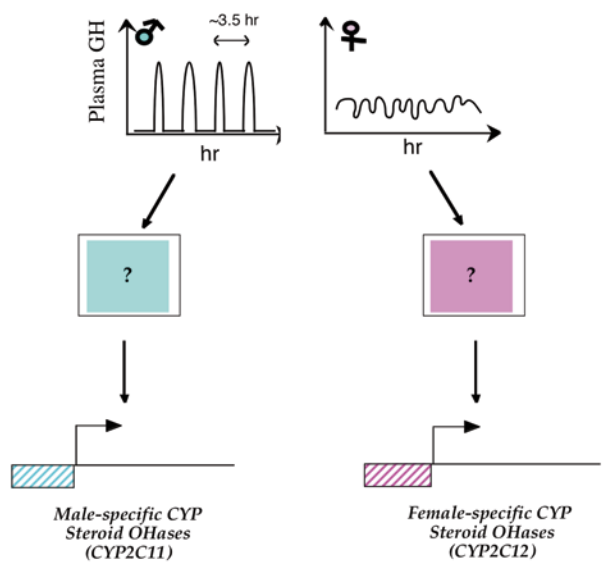


Fig. 11.6. Different growth hormone (*GH*)-induced intracellular signaling pathways are proposed to be activated by plasma GH pulses, leading to male-specific cytochrome P450 (*CYP*) expression (*left*), and by continuous GH stimulation, leading to female-specific *CYP* expression (*right*)

in activating JAK2, the GHR-associated tyrosine kinase that initiates downstream pathways of intracellular protein tyrosine phosphorylation. Investigation of the differential effects of the male versus female plasma GH pattern on nuclear protein tyrosine phosphorylation led to the discovery of an intracellular signaling protein and transcription factor, termed STAT5b (Fig. 11.5), that is intermittently present in its active, nuclear tyrosine-phosphorylated form in male liver but shows persistent nuclear activity in female liver [155]. STAT proteins are latent cytoplasmic transcription factors that are activated by tyrosine phosphorylation stimulated by a variety of cytokines and growth factors, and were first discovered as signal mediators that carry transcription signals into the nucleus in the interferon signaling pathway [156].

In hypophysectomized rat liver, where there is no endogenous GH signaling, there is little or no tyrosine-phosphorylated STAT5b protein in the nucleus; essentially all of the STAT5b protein is found in the cytosolic fraction, where it resides in a latent, inactive (nontyrosine-phosphorylated) form. However, when a hypophysectomized rat is injected with a single pulse of GH, STAT5b protein appears in the nucleus in its active, tyrosine-phosphorylated state within 10–15 min [155, 157]. This tyrosine phosphorylation reaction occurs on STAT5b tyrosine residue 699, enabling two STAT5b molecules to dimerize via mutual interactions between the phosphotyrosine residue on one STAT5b molecule and the SH2 domain (a protein module that recognizes and binds specifically to phosphotyrosine residues) on a second STAT5b molecule. The STAT5b–STAT5b dimer that is thus formed enters the nucleus rapidly, where it binds with high affinity to DNA sites upstream of genes that are transcriptionally activated in response to the initial GH stimulus (Fig. 11.5).

STAT5b is repeatedly activated by GHR/JAK2-catalyzed tyrosine phosphorylation in concert with the onset of each male plasma GH pulse. STAT5b thus undergoes repeated cycles of translocation from the cytoplasm into the nucleus, and then back out to the cytoplasm [155, 158]. For example, if the liver is excised from a rat killed at the time of a plasma GH pulse, then STAT5b is found to be tyrosine-phosphorylated and localized to the nucleus, whereas if the liver is excised from a rat killed at a time point between successive plasma GH pulses, STAT5b is inactive and cytoplasmic. This close temporal linkage between plasma GH pattern and the activation state of liver STAT5b has been confirmed in intact male rats killed at times shown to be specifically associated with spontaneous peaks or troughs of the plasma GH rhythm [159]. In contrast, in female rat liver, active, nuclear STAT5b

protein is detectable at essentially all points in time, albeit at a level that is generally much lower (~5–10%) than the peak male liver level [160]. Studies carried out in the mouse model show that liver STAT5 (primarily STAT5b) also shows intermittent activity when assayed across a panel of individual male livers, whereas in female mouse, liver STAT5 is active at all points in time—often at a level as high as that of male mouse liver [161]. Thus, the key difference between male and female liver is that STAT5b is intermittently activated by plasma GH pulses in males, but is persistently activated by the more continuous GH profile in females, as seen in both rats and mice [161, 162].

11.4.2.4.2 STAT5b Gene Knockout Mouse Model

Studies carried out in mice that are deficient in STAT5b (STAT5b-knockout mouse model) lend strong support to the proposal that STAT5b is an essential factor for sex-specific liver P450 gene expression [163] (see [164] for a review). Disruption of the *STAT5b* gene results in two striking phenotypes, both seen in STAT5b-deficient male but not female mouse liver. First, there is a global loss of GH-regulated, male-specific liver gene expression, including male-specific P450 gene expression. Second, the expression of several female-specific, GH-regulated liver P450 genes increases to near-normal female levels in livers of STAT5-deficient male mice, indicating negative regulation of the female-specific genes by STAT5b. STAT5a is unable to compensate for the loss of STAT5b [163, 165], but is essential for expression of a unique subset of female-biased genes in female liver [166]. The liver-enriched factor HNF4α cooperates with STAT5b in regulating liver sex-differences [56, 167].

These same phenotypes are seen in liver-specific STAT5a/STAT5b double knockout mice [168], but are not seen in mice where the disruption is limited to the STAT5a gene [163, 165], whose protein coding sequence is ~90% identical to that of STAT5b [169]. Not all sex-specific liver CYPs are dependent on STAT5b, however. Thus, continuous infusion of GH in male mice strongly induced (> 500-fold) the female-specific P450 gene *Cyp3a16* in both wild-type and hepatocyte STAT5ab-deficient male mouse liver, indicating that this sex-specific gene is subject to a STAT5a/STAT5b-independent mechanism of GH regulation [168]. Hypophysectomy and GH pulse replacement studies have established that these phenotypes of STAT5b-deficient mice are a direct response to the loss of STAT5b-dependent GH signaling in the liver, as opposed to indirect effects of the loss of STAT5b on the overall pattern of GH secretion by the pituitary gland [170].

11.4.2.4.3 Genome-Wide Mapping of Liver Binding Sites for STAT5 and Other GH-Regulated Transcription Factors

The strong, repeated pulses of GH-activated STAT5b that occur in adult male liver have been proposed to induce binding of STAT5b directly to STAT5 response elements found in promoters and other regulatory regions associated with STAT5 target genes, including sex-dependent P450 genes, stimulating gene transcription [155]. Consistent with this hypothesis, STAT5 response elements matching the consensus sequence TTC–NNN–GAA have been found upstream of several male-specific rat liver P450 genes, including CYPs 2C11, 2A2, and 4A2 [171]. GH-stimulated CYP promoter-luciferase reporter activity can be demonstrated in cell-based transfection experiments using the corresponding isolated STAT5 response elements, although the magnitude of the GH- and STAT5b-dependent gene induction is small, generally only ~2–3-fold [171, 172]. Moreover, although pulsatile STAT5b signaling is first seen in young male rats at ~5 weeks of age, when liver CYP2C11 expression is first detected, precocious activation of STAT5b, achieved in 3-week-old male rats given exogenous GH pulse injections, does not lead to precocious *CYP2C11* gene induction [158]. These and other findings suggest that STAT5b regulation requires a native chromatin environment (see below), as well as cooperative interactions with other factors, including liver-enriched transcription factors (HNFs) that work together with STAT5b to control the expression of sexually dimorphic liver P450 genes [171, 173–175].

Further insight into the sex-specific actions of GH-activated STAT5 was obtained by genome-wide mapping of liver binding sites for STAT5b and STAT5a (collectively, STAT5) using chromatin immunoprecipitation (ChIP-seq technology). These studies identified ~3500 sites spread throughout the genome that show strong sex-differential binding of STAT5 [161]. Male-biased STAT5 binding was shown to be enriched for nearby male-specific genes, and female-biased STAT5 binding was enriched for nearby female-specific genes. Mapping of the binding sites in mouse liver for BCL6, a GH- and STAT5-regulated male-biased repressor [161, 176], indicates that BCL6 enforces liver sex differences in male liver by preferentially binding to female-biased STAT5 binding sites that are nearby female-specific genes. This binding preference enables BCL6 to repress the expression of the STAT5-dependent female-biased genes in male liver [161] (Fig. 11.7). An analogous regulatory mechanism is operative in female liver, where Cux2, a female-specific repressor [57], represses ~35% of male-biased genes by preferentially binding to regulatory elements that are generally more open (more accessible) in male liver [58] (Fig. 11.7). In addition to these repressive actions of Cux2 on male-specific genes, Cux2 positively regulates ~35% of female-biased genes; however, most of these positive regulatory actions are not associated with direct Cux2 binding to the female-biased genes, and are thus likely to proceed by an indirect mechanism. Robust sex differences can thus be achieved for large numbers of sex-biased genes, including sex-biased *CYP* genes, by the complex interplay of multiple GH-regulated transcription factors (Fig. 11.7).

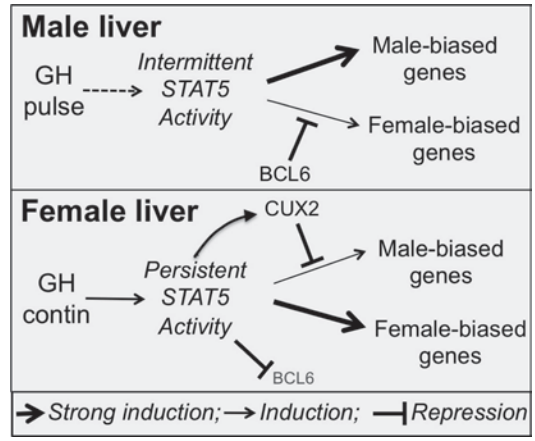


Fig. 11.7 Signal transducer and activator of transcription 5 (*STAT5*) is activated intermittently in male liver and more continuously (persistently) in female liver. Shown are the effects of *STAT5* and the transcriptional repressors Bcl6 (whose expression is male-biased) and Cux2 (whose expression is female-specific) on the activation and repression of sex-specific genes in mouse liver. *GH* growth hormone

11.4.2.4.4 GH Regulation of Chromatin States in Male and Female Liver

Changes in chromatin structure are a hallmark of epigenetic regulation and developmental plasticity and can be probed on a global scale using the enzyme deoxyribonuclease I (DNase I) to selectively cut open (accessible) chromatin sites (euchromatin) in freshly isolated intact liver nuclei (Fig. 11.8a). This technique, known as DNase hypersensitivity site (DHS) analysis, was employed to identify ~70,000 open chromatin regions across the entire genome in male and female mouse liver [177]. These DHS are expected to encompass four major classes of regulatory elements: promoters, enhancers, silencers, and insulators, and they encompass up to 90% of genome-wide binding sites for each of ten different liver transcription factors [177]. Importantly, more than 1200 of the 70,000 DHS showed robust, plasma GH-dependent differences in the extent of hypersensitivity between male and female mouse liver (Fig. 11.8b). The set of male-biased liver DHS was tenfold enriched for nearby male-specific genes compared to female-specific genes, and correspondingly female-biased DHS showed tenfold enrichment for being nearby female-specific genes. This finding is consistent with many of the sex-biased DHS serving as sex-dependent enhancers that positively regulate nearby sex-specific genes in mouse liver. Importantly, the above-described occurrence of sex-differential STAT5 binding, which is seen at many promoters and enhancers linked to sex-specific genes, shows very strong enrichment (up to 14-fold) for sex-biased DHS [161] (Fig. 11.9). Thus, sex differences in chromatin accessibility

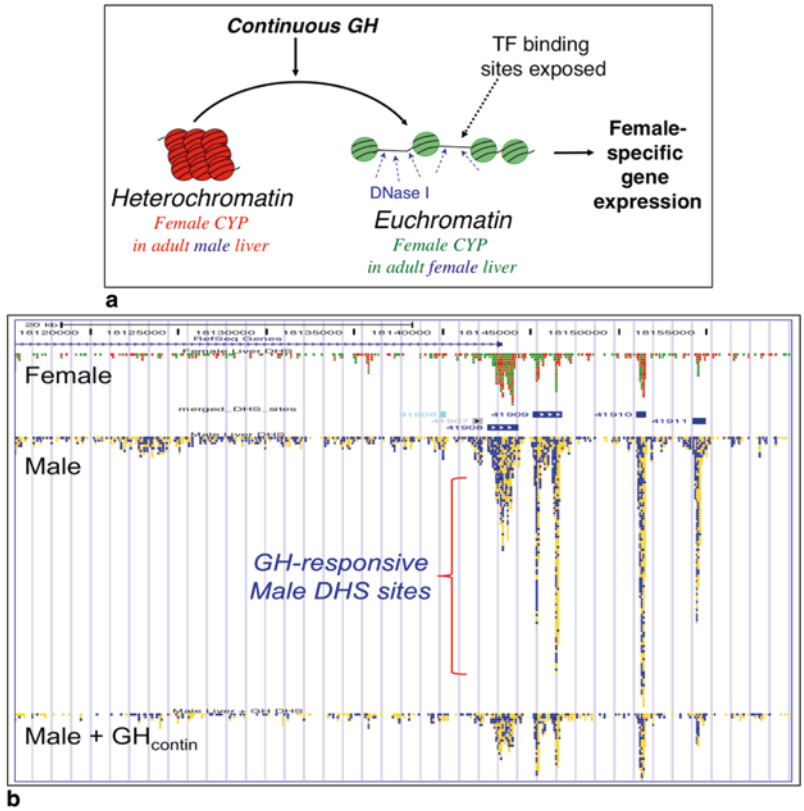


Fig. 11.8 DNase hypersensitivity assay for identification of open (accessible) chromatin regions as deoxyribonuclease (*DNase*) hypersensitive sites (*DHS*). **a** Schematic diagram indicating how continuous growth hormone (*GH*) treatment opens female-specific DNA regulatory regions (*DHS*) that are within closed (inaccessible) heterochromatin in male liver. Chromatin opening enables DNase to access the DNA backbone in intact liver nuclei and cleave (release) genomic DNA fragments, which are purified, sequenced, and mapped to the mouse genome. The figure is based on [18]. **b** Mapped DNA fragments released from adult female, adult male, and continuous GH-treated male mouse liver nuclei in the genomic region covering the 5′ end and upstream regulatory region of mouse *Cyp7b1,* which is ~9-fold more highly expressed in male than female mouse liver. The figure shows five distinct chromatin regions nearby *Cyp7b1* that are much more accessible (larger peaks of released DNase fragments in male than female liver; middle track) and are partially closed down to the normal female level following continuous GH treatment for 7 days. Data are based on [177]

are regulated by plasma GH patterns and appear to be a key feature of sex-differential gene expression. However, many sex-specific DHS are distant from sex-specific genes (60 % are > 1 million bp away from the nearest sex-specific gene) [177], suggesting regulation occurs from a distance via chromatin loops, which complicates efforts to identify gene targets of the sex-specific genes and their underlying mechanisms of GH regulation.

Further complexity is indicated by the finding that sex differences in the liver emerge at puberty [53], when sex-specific genes are subject to either positive regulation (class I genes) or negative regulation (class II genes) by pituitary GH [123, 124] (Fig. 11.4). Furthermore, many sex-specific CYP genes respond slowly (over days) to a change in plasma GH status (Fig. 11.10) [124, 167], even though STAT5 binds to these genes within minutes after its activation by a plasma GH pulse [161]. This suggests that the sex-dependent actions of GH and STAT5 are dependent on slower, secondary events, including chromatin modifications or other sex-dependent

Fig. 9

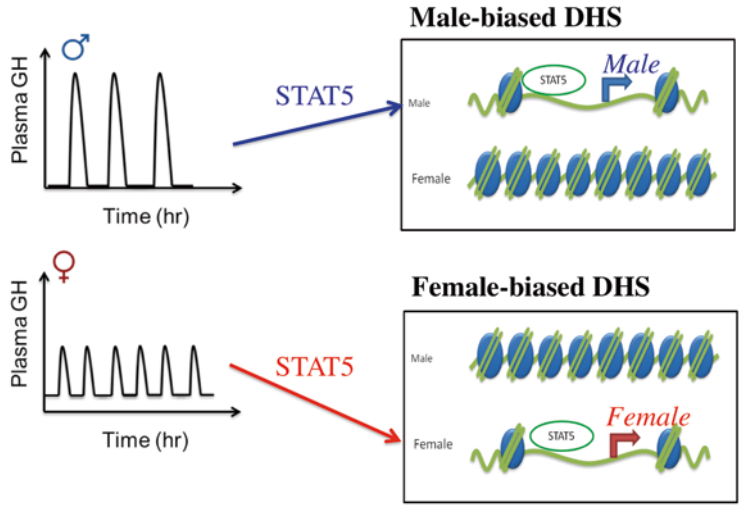


Fig. 11.9 Sex-differential binding of signal transducer and activator of transcription 5 (*STAT5*) to liver chromatin in male and female liver. The male plasma growth hormone (*GH*) profile activates a male-specific pattern of STAT5 binding to liver chromatin at sites that are more accessible in male than female liver ("male-biased DHS"), as determined by DNase hypersensitivity site (*DHS*) analysis as described in Fig. 11.8, leading to transcriptional activation of a nearby male-specific gene. Correspondingly, the female plasma GH profile activates a female-specific pattern of STAT5 binding to liver chromatin at sites that are more accessible in female than male liver ("female-biased DHS"), leading to transcriptional activation of a nearby female-specific gene

Fig. 10

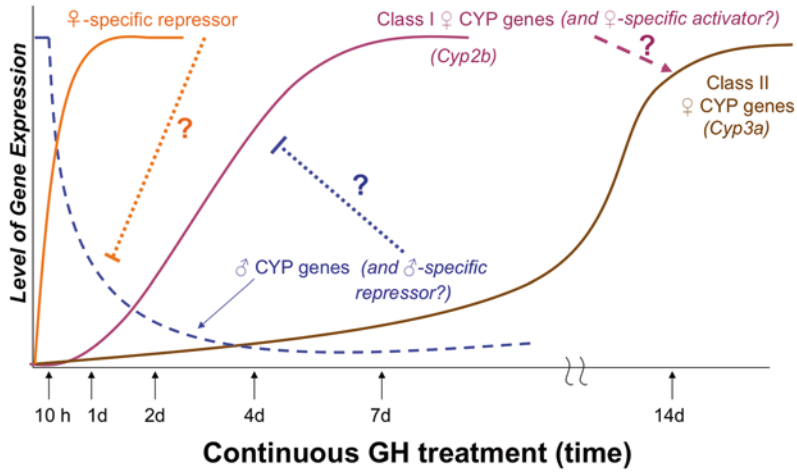


Fig. 11.10 Hierarchical changes in the expression of sex-specific genes in male mouse liver following continuous growth hormone (*GH*) treatment assayed at time points ranging from 10 h to 14 days. Results based on studies reported in [167]. Hypothetical relationships between induced and repressed genes are marked by dashed lines and question marks. Female-specific repressors, such as Cux2, are rapidly activated in livers of male mice given GH by continuous infusion. These repressors are proposed to downregulate many male-specific genes, including some genes that serve as repressors of female-specific genes, such as *Cyp2b9,* which are derepressed. The derepression of other female-specific genes, including certain *Cyp3a* genes, is substantially delayed in continuous GH-infused male mouse liver. The figure is based on [18]. *CYP* cytochrome P450

epigenomic changes [178]. Key unanswered questions relate to the mechanisms controlling these sex differences in liver chromatin states: How are these sex-differential states established (presumably this occurs at puberty), how are they maintained by the sex-differential plasma GH profiles, and what are the roles of the sex-dependent patterns of liver STAT5 activation—intermittent STAT5 activation in male liver and persistent STAT5 activation in female liver—in these processes?

11.4.2.4.5 Downregulation of Hepatic STAT5b Signaling

Other questions relating to GH and the STAT5b signaling pathway that are of current research interest include how the cycle of STAT5b activation is turned off at the conclusion of each GH pulse, and how STAT5b is subsequently returned to the cytoplasm in an inactive form, where it apparently waits for ~2–2.5 h until it can be reactivated by the next pulse of GH (Fig. 11.5) [179]. These events may, in part, involve a family of inhibitory proteins, referred to as SOCS and CIS proteins, which turn off signals to various hormones and cytokines, including GH [180, 181]. In the case of GH signaling, SOCS proteins bind to the GHR–JAK2 tyrosine kinase complex, and thereby inhibit GH signaling by a complex series of interrelated mechanisms [182]. CIS may be induced to a higher level by the continuous (female) GH pattern than by the pulsatile (male) GH pattern and has been implicated in the downregulation of GH-induced STAT5b signaling in liver cells exposed to the female GH pattern [182].

11.4.3 Regulation by Thyroid Hormone

11.4.3.1 Cytochromes P450

Although GH is the major regulator of sex-specific liver P450s, thyroid hormone also plays a critical role. The major thyroid hormones, T3 and T4, positively regulate some [47, 110] but not all [48] female-predominant liver P450 enzymes, while they negatively regulate several of the male-specific enzymes [183, 184] (Table 11.1). These effects of thyroid hormone are operative at the mRNA level, and are independent of the indirect effects that thyroid hormone has on liver P450 levels as a consequence of its effects on liver GHRs [185] and its stimulation of GH gene transcription and GH secretion by the pituitary [186].

11.4.3.2 NADPH-CYP Reductase

Thyroid hormone is also required for expression of NADPH-CYP reductase, a flavoenzyme that catalyzes electron transfer to all liver microsomal P450 enzymes. P450 reductase is an obligatory, and often rate-limiting electron-transfer protein that participates in all microsomal P450-catalyzed drug oxidation and steroid hydroxylase reactions [187, 188]. This thyroid hormone dependence of P450 reductase enzyme expression is evidenced by the major decrease (>80% reduction) in liver microsomal P450 reductase activity and P450 reductase mRNA levels that occurs following hypophysectomy [189] or in response to methimazole-induced hypothyroidism [190]. It is further supported by the reversal of this activity loss when thyroxine (T4), but not GH or other pituitary-dependent factors, is given at a physiologic replacement dose [189, 190]. Restoration of liver P450 reductase activity in vivo by T4 replacement also effects a substantial increase in liver microsomal P450 steroid hydroxylase activities. A similar effect can be achieved when liver microsomes isolated from hypophysectomized rats are supplemented with exogenous, purified P450 reductase, which preferentially stimulates steroid hydroxylation catalyzed by microsomes prepared from thyroid-deficient animals [189]. The induction of rat hepatic P450 reductase in livers of rats treated with exogenous thyroid hormone occurs by transcriptional [191] and post-transcriptional mechanisms [192] and appears to involve enhanced protein stability in hyperthyroid rat liver [193]. P450 reductase levels are also modulated by thyroid hormone status in several extrahepatic tissues [190]. Interindividual differences in P450 reductase activity could occur in response to physiological or pathophysiological differences in circulating thyroid hormone levels

and may be an important contributory factor to individual differences in P450 reductase/CYP-catalyzed procarcinogen bioactivation.

11.5 Alteration of Sex-Dependent Liver P450 Expression by Hormonal Perturbation

Circulating hormones levels can be altered in response to drug therapy; exposure to various xenobiotics; disease states such as diabetes mellitus, liver cirrhosis and steatosis, and kidney failure; dietary factors; pregnancy; and lactation. The resultant changes in circulating hormone levels or alterations in hormone secretory dynamics could influence the expression of specific liver P450s. The following sections describe some of the factors that are known to cause hormonal perturbation and discuss the impact of these changes on sex-dependent liver P450 expression and on P450-dependent drug and xenobiotic metabolism and toxicity.

11.5.1 Xenobiotics

11.5.1.1 Drugs

Liver P450 enzyme profiles are altered in rats treated with the anticancer drugs cisplatin [194, 195], cyclophosphamide [82, 196], and ifosfamide [196] by mechanisms that involve hormonal perturbations that these cytotoxic agents induce. Treatment of adult male rats with a single dose of cisplatin depletes serum testosterone. This effect persists for up to 28 days after cisplatin administration and is associated with feminization of hepatic liver enzyme expression [194]. Thus, cisplatin-treated male rats have elevated levels of the female-predominant enzymes CYP2A1, CYP2C7, and steroid 5α-reductase, but have reduced levels of the male-specific CYP2A2, CYP2C11, and CYP3A2 [194, 195]. The effects of cisplatin on circulating androgen levels may result from the drug's action on the testes [197, 198]; however, effects on the hypothalamus also appear to contribute, both to the depletion of circulating testosterone and to the alteration in liver P450 expression [194]. Cisplatin treatment of adult female rats severely decreases circulating estradiol levels and significantly reduces the expression of the estrogen-dependent CYP2A1, CYP2C7, and CYP2C12 [195].

Serum testosterone is also depleted in adult male rats treated with cyclophosphamide [82, 196, 199] or ifosfamide [196], and this depletion is associated with feminization of liver enzyme profiles [82, 196] in a manner similar to that produced by cisplatin. While endogenous testosterone secretion can be stimulated in cyclophosphamide-treated rats by the luteinizing hormone analogue chorionic gonadotropin, the resultant increase in serum testosterone does not reverse the loss of hepatic CYP2C11 expression [82]. This result is analogous to the earlier finding that the suppression of CYP2C11 by 3,4,5,3′,4′,5′-hexachlorobiphenyl [200] is not causally related to the associated depletion of serum testosterone [201]. The alteration of liver enzyme expression by cyclophosphamide may therefore involve action at the hypothalamic–pituitary axis, which establishes the sex-dependent plasma GH profile that in turn dictates the expression of CYP2C11 and other sex-dependent liver P450 enzymes, as discussed earlier in this chapter. CYP2C11 can also be suppressed by other mechanisms, as demonstrated by the finding that CYP2C11 levels are suppressed by the anticancer drug 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU; lomustine) without affecting circulating testosterone levels [202]. Conceivably, CCNU may act directly on the hypothalamic–pituitary axis to alter key signaling elements in the ultradian rhythm of circulating GH.

Other drugs that suppress liver CYP2C11 and CYP3A2 levels include cyclosporine [203, 204] and chloramphenicol [205], although the latter effects are strain dependent and are associated with a modest reduction in plasma levels of thyroxine but not testosterone [205]. GH does not appear to play a role in the suppression of CYP2C11 and CYP3A2 by cyclosporine, which does not alter the plasma GH peak amplitude, number, or duration [206]. Phenobarbital [24, 207, 208], dexamethasone [28], 5-fluorouracil [209], doxorubicin [210], fenofibrate [211], rosuvastatin [212],

and neuroleptics such as levomepromazine, perazine, and thioridazine [213] also reduce hepatic CYP2C11 expression, but the underlying mechanisms have not been determined.

As discussed above, the anticancer drug cisplatin provides an example of a foreign chemical that depletes serum testosterone and consequently feminizes the expression of liver P450s in adult male rats. This type of alteration in the profile of liver P450 enzymes could have important pharmacological consequences, as suggested by the finding that cisplatin suppression of CYP2C11 decreases liver P450-catalyzed activation of anticancer prodrugs, such as cyclophosphamide [195, 214, 215] and ifosfamide [215]. Liver P450 activation of these latter two drugs is required for their anticancer drug activity [216], and CYP2C11 contributes significantly to this metabolic pathway in adult male rat liver [214, 215]. Clinical studies indicate that cisplatin may exert effects on circulating hormone levels in human cancer patients that are similar, though not identical, to those seen in rats [217]. If these hormone perturbations alter P450 enzyme levels in human liver, this could have an impact on drug–drug interactions in patients given cisplatin in combination with anticancer drugs such as cyclophosphamide.

11.5.1.2 Ethanol

Adult male rats administered ethanol by a total enteral nutrition system have reduced hepatic CYP2C11 and CYP3A2 levels, whereas their CYP2A1 activity is unaltered [218]. The same ethanol treatment alters the dynamics of plasma GH secretion by decreasing the GH pulse amplitude and increasing the GH pulse frequency. The increased frequency of GH pulses can thus explain the reduced expression of CYP2C11 after chronic ethanol intake because hepatocytes require a minimum "off time" to express the male pattern of GH secretion that stimulates CYP2C11 expression [86]. In another study, chronic intragastric infusion of ethanol-containing diets suppressed CYP3A2 while substantially increasing the expression of CYP3A9 in adult male rats [219].

11.5.1.3 Aromatic Hydrocarbons

Exposure of adult male rats to an aromatic hydrocarbon suppresses hepatic CYP2C11 mRNA, protein, and activity [220]. Aromatic hydrocarbons that downregulate CYP2C11 include 3-methylcholanthrene (3MC) [24, 26, 200, 208, 221–223]; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) [224]; anthracene and its derivatives, including benz(*a*)anthracene, dibenz(*a, c*)anthracene, dibenz(*a, h*)anthracene, and 7,12-dimethylbenz(*a*)anthracene [225]; ethylbenzene [226–230]; and Sudan III [231]. In the case of 3MC, this suppression reflects a decrease in the rate of CYP2C11 transcription [232]. The mechanisms by which aromatic hydrocarbons alter CYP2C11 expression are not well understood; however, 3MC [200] and 2,3,7,8-TCDD [233] have been reported to decrease serum testosterone levels. In hypophysectomized adult male rats, 3MC interferes with the stimulation of CYP2C11 expression by GH [234], but in a manner that does not involve STAT5b [235]. The hormonal basis for CYP2C11 suppression by ethylbenzene has also been investigated. Treatment of intact adult male rats with ethylbenzene decreases hepatic CYP2C11 expression, as assessed at the level of mRNA, protein, and enzyme activity (testosterone 2α-hydroxylation) [226–230]. This appears to reflect an alteration in plasma GH profile by ethylbenzene because it does not decrease hepatic CYP2C11 expression in hypophysectomized adult male rats administered twice-daily s.c. injections of GH [230], which mimics the male plasma GH pattern [86]. The molecular mechanism of CYP2C11 suppression by aromatic hydrocarbons has been investigated. Results from in vitro binding experiments and luciferase reporter assays conducted in cell culture models suggest that the aryl hydrocarbon (Ah) receptor is responsible for the suppression of rat hepatic CYP2C11 by 3MC [220]. 2,3,7,8-TCDD also decreases, albeit to a lesser extent, hepatic expression of the male-specific, mouse liver steroid 16α-hydroxylase Cyp2d9 [236], which is known to be regulated by the pulsatile male pattern of GH secretion in a manner that is dependent on Stat5b [163, 170, 237]. Experiments performed on AhR-knockout

mice indicate that the suppression of Cyp2d9 by 2,3,7,8-TCDD is AhR dependent, and it occurs by disrupting the GHR–JAK2–STAT5b signaling pathway [238].

11.5.2 Pathophysiologic State

11.5.2.1 Diabetes Mellitus

Uncontrolled insulin-dependent diabetes is not only accompanied by defective carbohydrate metabolism, which results in hyperglycemia, hyperlipidemia, and hyperketonemia, but also associated with hormonal changes, including a reduction in circulating testosterone [239–241], thyroid hormone, and plasma GH [242, 243]. As discussed above, these hormones regulate many liver P450 enzymes, either directly or indirectly. Accordingly, the diabetic state is associated with profound changes in the levels of several hepatic P450 enzymes, whereas diabetes leads to induction of several rat liver P450s, including CYP1A [244], CYP2A1 [243, 245], CYP2B1 [244, 246–248], CYP2C7 [245], CYP2E1 [248–252], CYP4A2 [245], and CYP4A3 [245], while it suppresses CYP2A2 [243], CYP2C11 [243, 244, 246–248, 252], and CYP2C13 [243]. Changes in the levels of some of these liver P450s (e.g., CYP2C11 and CYP2E1) have been shown at the mRNA level and are reversed by insulin replacement [246, 247, 253, 254].

The profile of GH secretion in the diabetic male rat is altered so as to resemble the pattern found in the normal female rat [242]. The induction of CYP2A1 and CYP2C7 in diabetic male rats can therefore be explained, at least in part, as a response to the more continuous pattern of GH secretion, which stimulates expression of these P450 forms [35, 76, 110, 255]. In contrast, this pattern of GH secretion reduces CYP2A2 and CYP2C13 levels because continuous GH administration suppresses these two P450s [35, 37, 129]. CYP2C11 expression is obligatorily dependent on the intermittent male pattern of plasma GH secretion [86]. Therefore, the more continuous secretion of GH in diabetic male rats [242] would be expected to suppress this P450. In the case of CYP2B1, GH pulse height is the suppressive signal [121] and, accordingly, the reduction in GH peak concentration in diabetic male rats [243] leads to increases in CYP2B1 levels [247, 254]. A GH-independent mechanism is likely to contribute to some of the other effects of diabetes on liver P450 levels. GH, independent of its plasma profile, is suppressive toward hepatic CYP2E1 expression [75], but the levels of this P450 are substantially elevated in both diabetic male and female rats [243, 256, 257]. The induction of CYP2E1 in diabetes has been attributed to increased plasma concentrations of ketone bodies [250, 258]. A role of hypoinsulinemia and hyperglucagonemia has been proposed for the suppression of CYP2C11 in diabetes, based on the finding that treatment of cultured rat hepatocytes with glucagon decreases CYP2C11 expression in a cyclic adenosine monophosphate (cAMP)-dependent manner and this decrease can be reversed by insulin administration [259].

Diabetes mellitus is associated with a decrease in P450-mediated in vitro hepatic metabolism of imipramine [241, 260], lidocaine [241], codeine [261], and chlorpromazine [261]. In addition, alteration of liver P450 expression in diabetes is postulated to be responsible for the enhanced in vitro metabolic activation of certain chemical carcinogens, including Try-P-1(3-amino-1,4-dimethyl,5H-pyrido(4,5-b)indole) and Try-P-2(3-amino-1-methyl-5H-pyrido(4,3-b)indole) [262]. These examples demonstrate the potential for alterations in liver P450 expression that potentially lead to reduced drug metabolism and enhanced procarcinogen bioactivation.

11.5.2.2 Liver Disease

While certain P450 enzymes (e.g., CYP1A2, CYP2E1, and CYP3A forms) are known to play a role in the pathogenesis of various liver diseases [263], studies in experimental models of liver disease have shown that liver cirrhosis and steatosis impact the expression of sex-dependent liver P450 enzymes. Adult male rats fed a chronic choline-deficient diet to induce cirrhosis have increased serum estradiol concentrations [264] and decreased testicular weight [265] and serum testosterone levels [264], indicating gonadal abnormalities occur in liver cirrhosis. In asso-

ciation with the perturbation in hormonal status is a major decline in hepatic CYP2C11 content [264], and this decline is not accompanied by an increase in hepatic steroid 5α-reductase activity [266]. The suppression of hepatic CYP2C11 in adult male rats is also evident in other models of liver cirrhosis, including bile duct ligation [267, 268], carbon tetrachloride-induced cirrhosis [266, 268, 269], and *N*-dimethylnitrosamine-induced cirrhosis [244]. It remains to be determined whether the alteration in serum steroid hormone levels contributes to the apparent demasculinization of liver P450 profiles in these models of liver cirrhosis. CYP2C11 is also suppressed in adult male rats treated with orotic acid [270] or clozapine [271] to induce liver steatosis. However, the mechanism for CYP2C11 suppression in these experimental models of liver steatosis is not known.

11.5.2.3 Kidney Disease

Kidney disease affects the pharmacokinetics of many drugs, including drugs that are cleared by nonrenal elimination pathways, including hepatic metabolism [272]. Experimental models of acute kidney failure are associated with a decrease in total liver P450 content [273, 274] and decreased hepatic expression of CYP2C11 [275, 276] and CYP3A2 [276] in adult male rats. These decreases are also seen in chronic kidney failure, as elicited by a two-stage 5/6 nephrectomy protocol in adult male rats [277–280]. An inverse exponential correlation exists between serum creatinine concentration and hepatic expression of CYP2C11 and CYP3A2, indicating that disease progression influences in an exponential manner the extent of suppression of the male-specific liver P450 enzymes [281]. Analysis of serum from rats [282] or human patients [283] with chronic kidney failure suggests that uremic toxins contribute to the reduced expression of liver CYP2C11 and CYP3A2. Limited information is available on the hormonal basis for the suppression of these male-specific liver P450 enzymes in kidney failure. Neonatal nephrectomy abolishes the typical pulsatile male pattern of GH secretion in adult male rats so that their plasma GH profile resembles the more continuous plasma GH profile in adult female rats [284]. In neonatally nephrectomized adult male rats, the female pattern of GH profile is accompanied by increased hepatic expression of the female-specific CYP2C12 along with the suppression of the male-specific hepatic CYP2C11 [284].

11.5.3 Dietary Factors

Specific dietary constituents may also influence the expression of sex-dependent liver P450s and other enzymes [285]. In adult male rats, dietary vitamin A deficiency reduces hepatic CYP2C11 [286–288] and CYP4A2 levels [288] and induces steroid 5α-reductase activity [289]. These effects are accompanied by a decrease in serum testosterone levels [287]. The decrease in hepatic CYP2C11 but not CYP4A2 protein expression in rats on a vitamin A-deficient diet can be restored by exogenous administration of methyltrienolone (a synthetic androgen) to levels comparable to those in rats fed a vitamin A-adequate diet [288, 290]. Twice-daily s.c. administration of GH, which induces CYP2C11 expression in hypophysectomized male rat liver [86], does not restore the expression of CYP2C11 or CYP4A2 in male rats fed a vitamin A-deficient diet [288].

Dietary trace minerals can also alter the liver expression of sex-dependent P450 enzymes. Prepubertal male rats fed a zinc-deficient diet during the pubertal period have depleted serum testosterone levels and a feminized pattern of hepatic mRNA expression, as evidenced by a reduction in CYP2C11, CYP3A2, and CYP3A18 and by an elevation in CYP2C12 and CYP3A9 [291]. However, the precise neuroendocrine mechanisms responsible for the feminization of hepatic P450 enzyme expression by dietary zinc deficiency remain to be elucidated.

Finally, caloric restriction and food deprivation have been shown to modulate hepatic expression of sex-dependent liver steroid-metabolizing enzymes, including CYP2C11 [292], which is suppressed, and CYP3A9 [293] and steroid 5α-reductase [292], which are induced. These are situations in which glucagon levels are high and

insulin levels are low, analogous to the diabetic state (see Sect. 11.5.2.1).

11.5.4 Pregnancy and Lactation

Pregnancy is associated with major physiological changes, including increases in the maternal circulating levels of estrogen and progesterone [294]. However, rodent studies have indicated that pregnancy is not associated with alteration in maternal hepatic expression of the female-specific CYP2C12 in rats [295, 296] or Cyp2b9 in mice [297]. Similarly, lactation is not associated with any changes in CYP2C12 expression in maternal rat liver [298].

11.6 Effect of Estrogen and Progesterone on Expression of Xenobiotic-Inducible Liver P450 Enzymes

The liver expresses P450s that are subject to nuclear-receptor-mediated induction by various chemicals, including many structurally diverse drugs and other xenobiotics. Enzymes in the CYP2B and CYP3A subfamily are examples of major xenobiotic-inducible mammalian liver P450s. Induction of CYP2B and CYP3A is under the primary control of the constitutive androstane receptor (CAR) and pregnane X receptor (PXR), respectively [299]. Emerging evidence indicates that steroid hormones, such as estrogen and progesterone, are capable of increasing the expression of several of the xenobiotic-inducible liver P450 enzymes and activating specific receptors involved in P450 induction. Interested readers should refer to Chap. 10 for a detailed discussion on receptor-mediated induction of P450 enzymes.

11.6.1 Estrogen

Plasma levels of estrogens in nonpregnant women are usually at a low nanomolar concentration [300], but rises during pregnancy, reaching a peak of low micromolar concentration by the third trimester [301]. Treatment of primary cultures of human hepatocytes with 17β-estradiol (1 μM for 72 h) increases the levels of CYP2B6 mRNA [302–304] and CYP2B6 enzyme activity, as assessed by (*S*)-mephenytoin *N*-demethylation [303] and bupropion hydroxylation [302, 304]. Dose–response data indicate that 17β-estradiol increases human hepatocyte CYP2B6 expression with an EC_{50} of 2 μM and an E_{max} of 34-fold increase over vehicle control [304]. As shown in hepatocyte samples from the same donors, these values are comparable to those reported for rifampicin [304], which is a known inducer of CYP2B6 [305]. Consistent with the induction of CYP2B6 by 17β-estradiol in primary cultures of human hepatocytes [302–304], pregnancy is associated with an increase in the clearance of methadone [306, 307], which is metabolized primarily by CYP2B6 [308]. Molecular studies showed that 17β-estradiol (1 μM) activates human CAR, as assessed in a cell-based reporter gene assay, and induces nuclear translocation of CAR [302]. Transactivation of both CAR and estrogen receptor α (ERα) leads to a synergistic increase in CYP2B6 expression, suggesting that CYP2B6 induction by 17β-estradiol depends on the concentration of the steroid hormone and involves more than one receptor signaling pathway. At low concentrations (<0.1 μM), 17β-estradiol induces CYP2B6 by activating ERα, whereas at higher concentrations (≥0.1 μM), it induces CYP2B6 by activating both CAR and ERα [302]. Induction of mouse Cyp2b10 [309–312] and activation of mouse CAR [310, 312, 313] by micromolar concentrations of 17β-estradiol have also been reported. In addition to CYP2B6, 17β-estradiol (1 μM for 72 h) is also able to induce CYP2A6 in primary cultures of human hepatocytes [303]. Consistent with this finding, pregnancy is associated with an increase in the clearance of nicotine [314], which is metabolized primarily by CYP2A6 [315]. 17β-Estradiol (1 μM for 72 h) also increases, but only minimally, CYP3A4 expression in primary cultures of human hepatocytes [303]. At this concentration, 17β-estradiol does not activate PXR [316–318], which is a major regulator in the induction of CYP3A4 [316, 317, 319].

11.6.2 Progesterone

The plasma level of progesterone in nonpregnant women is ~1 nM and increases up to 400 nM in the third trimester of pregnancy [300]. At micromolar concentrations, progesterone (1–10 μM for 72 h) minimally increases the expression and activity of CYP2B6, CYP3A4, and CYP3A5 in primary cultures of human hepatocytes [303]. The induction of these P450s by progesterone is likely the result of activation of PXR, which is activated by progesterone at 10 and 50 μM [316, 317, 319]. Progesterone (1 and 10 μM) does not influence human CAR activity [320, 321]. In contrast, progesterone (3 and 10 μM) has been reported to decrease mouse CAR activity, as determined in a cell-based reporter gene assay [310, 313], suggesting it is an inverse agonist of mouse CAR.

11.7 Conclusion

Complex sex-dependent expression patterns characterize a subset of liver P450 enzymes, which impart sex differences in xenobiotic metabolism, pharmacokinetics, and toxicity, as seen in rats, mice, and other species, including humans. The temporal pattern of pituitary GH secretion differs between the sexes, inducing sex-dependent transcriptional events associated with sex-dependent epigenetic changes and chromatin states, and is a key factor in the regulation of the sex-dependent liver P450 enzymes. A pulsatile plasma GH profile, characteristic of adult male rats and mice, induces repeated activation of tyrosine kinase signaling from the GHR to the transcription factor STAT5b. GH-activated STAT5b translocates into the nucleus, where it binds to chromatin in a sex-biased manner, enabling it to regulate the transcription of sex-dependent liver P450 genes. In female liver, continuous or near-continuous exposure to GH leads to persistent STAT5 signaling to the nucleus, in contrast to the intermittent signaling that occurs in male liver. STAT5b-dependent liver gene expression is modulated by cooperative interactions with liver-enriched transcription factors, and with the sex-dependent, GH-regulated transcriptional repressors BCL6 (male-biased) and Cux2 (female-specific). Thyroid hormone is an important regulator of liver metabolic function and can act directly, by influencing the expression of individual P450 enzymes, as well as indirectly, via its stimulatory effects on hepatic NADPH-P450 reductase activity. Gonadal steroids impact sex-dependent liver P450s indirectly, via their effects on the hypothalamic–pituitary axis and its control of the sex-dependent plasma GH profiles. The hormone-regulated expression of sex-dependent liver P450s can be altered by diverse factors, including exposure to drugs and other xenochemicals, pathophysiologic states, and dietary factors, with effects on P450-catalyzed drug metabolism and carcinogen activation. Sex steroids, notably estrogen and progesterone, can increase the expression of the sex-dependent human hepatic P450 enzyme CYP3A4 by activating pregnane X receptor (PXR), which regulates the expression of genes involved in a broad array of biological processes, including transport and metabolism of endogenous substances and xenobiotics.

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P450 Enzymes in Steroid Processing

12

Richard J. Auchus and Walter L. Miller

Abbreviations

ACTH	Adrenocorticotropic hormone (Corticotropin)
AKR	Aldo-Keto reductase
CAH	Congenital adrenal hyperplasia
CRPC	Castration-resistant prostate cancer
DHEA	Dehydroepiandrosterone
11DOC	Deoxycorticosterone
FDX	Ferredoxin
FDXR	Ferredoxin reductase
FSH	Follicle-stimulating hormone
17OHP	17-Hydroxyprogesterone
HSD	Hydroxysteroid dehydrogenase
3βHSD	3β-Hydroxysteroid dehydrogenase/$\Delta^5 \rightarrow \Delta^4$-isomerase
ILD	Isolated 17,20-lyase deficiency
KIE	Kinetic isotope effect
LH	Luteinizing hormone
17OH-Allo	5α-pregnane-3α, 17α-diol-20-one (17-hydroxyallopregnanolone)
POR	P450 Oxidoreductase
SDR	Short-chain dehydrogenase-reductase
StAR	Steroidogenic acute regulatory protein
ZF	Adrenal zona fasciculata
ZG	Adrenal zona glomerulosa
ZR	Adrenal zona reticularis

R. J. Auchus (✉)
Division of Metabolism, Endocrinology, and Diabetes, Department of Internal Medicine, University of Michigan Medical Center, 5560A, MSRBII 1150 W. Medical Center Drive, Ann Arbor, MI 48109, USA
e-mail: rauchus@med.umich.edu

W. L. Miller
Division of Endocrinology, Department of Pediatrics, University of California, 513 Parnassus Ave, Box 1346, San Francisco, CA 94143-1346, USA
e-mail: wlmlab@ucsf.edu

12.1 Introduction

Steroid hormones were first defined by their biological activities prior to their structural elucidation. Structures determined by classic organic chemistry showed that all steroids possess the cyclopentanoperhydrophenanthrene hydrocarbon frame as in cholesterol, and that different types of steroids differed mainly in the number of carbon atoms and the oxidation state of specific carbon atoms. In particular, the presence of hydroxyl or ketone groups at carbons 3, 11, 17, and 21, largely correlate with biologic activity. Reichstein painstakingly deduced the structures of multiple steroids found in bovine adrenals; while these suggested precursor–product relationships, it was not until the various human disorders of steroidogenesis were studied that the steroidogenic pathways become clear.

The discovery of P450 enzymes occurred in stages: Martin Klingenberg was probably first to report the classic CO-induced difference spectrum with a peak at 450 nm [1], and Omura and Sato then described the quantitative use of this spectrum and were the first to use the term "cytochrome P450" in print [2]. David Cooper and Otto Rosenthal, in collaboration with Ron

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Estabrook, demonstrated the involvement of cytochrome P450 enzymes in steroid biochemistry when they showed that carbon monoxide inhibited the 21-hydroxylase activity in adrenal microsomes [3]. Radiolabeled steroids became commercially available in the 1960s, which facilitated experiments to confirm precursor–product relationships. Despite these initial advances, further progress was slow, due to low abundance of enzymes, the need to obtain animal adrenals as the source, the tedious nature of the assays, species-specific variations in the pathways, and the inability to purify the enzymes, which limited the interpretation of messy experiments.

Additional landmark discoveries followed, including the demonstration of cholesterol side-chain cleavage activity in adrenal mitochondria [4] and the demonstration that this activity was catalyzed by a P450 [5, 6], the identification of aromatase activity in placental microsomes [7], and the purification of 17-hydroxylase and 17,20-lyase activities in a single protein from pig testis [8–10]. The advent of molecular biology led to the cloning of the steroid hydroxylase cDNAs and genes, as well as the characterization of mutations in these genes causing human disease (for review, see [11, 12]). Unlike xenobiotic metabolism, steroidogenesis requires several P450 enzymes and activities to produce active hormones. As the general principles of P450 chemistry have been covered in other chapters, we will begin with a discussion of these pathways and then cover the individual enzymes.

12.1.1 Steroid Classes and Receptors

Although steroid hormones are classified primarily according to their biological activities rather than their structures (Table 12.1), some general structure–activity correlations exist for the major endogenous steroids. For example, androgens and estrogens account for the masculinizing and feminizing products of the testis and ovary, respectively. Endogenous androgens contain 19 specific carbon atoms, and endogenous estrogens contain 18 carbon atoms, lacking one specific methyl group found in androgens. All other major classes of steroid hormones contain the same scaffold of 21 carbon atoms. Progesterone from the ovarian corpus luteum enables implantation of the fertilized ovum and maintenance of pregnancy. The placenta makes progesterone in the latter half of gestation and completes the synthesis of estrogen throughout pregnancy. The adrenal cortex contains three zones, each with unique repertoires of enzymes and thus distinct major products. The outermost zona glomerulosa (ZG), a thin layer a few cells thick, produces the mineralocorticoid aldosterone, which regulates sodium and fluid balance. The zona fasciculata (ZF), located beneath the ZG, produces glucocorticoids, which mediate a host of actions: promoting response to stress, increasing glucose production from the liver, suppressing the immune response, and stimulating lipolysis. The innermost zone of the adrenal cortex is the zona reticularis (ZR), which is essentially found only in primates and makes abundant 19-carbon precursors of androgens, but minimal biologically active androgen

Table 12.1 Steroid hormones

Class	Steroids	Bioactivity	Needed P450s
Androgen	Testosterone	Masculinizing	scc, c17
	Dihydrotestosterone	Masculinizing	scc, c17
Estrogen	Estradiol	Feminizing	scc, c17, aro
	Estriol	Feminizing	scc, c17, aro, 3A7
Progestin	Progesterone	Maintain pregnancy	scc
Mineralocorticoid	Aldosterone	Sodium balance	scc, c21, c11AS
Glucocorticoid	Cortisol	Stress response	scc, c17, c21, c11β
	Corticosterone	Stress response	scc, c17, c21, c11β
Vitamin D	Calcitriol	Calcium balance	2R1, c1α, c24

steroids. Vitamin D, a sterol hormone in which the B ring of the core cyclopentanophenanthrene structure has been opened, retains the side chain of cholesterol and has 27 carbons.

Each steroid hormone acts principally by binding a cognate nuclear receptor. In most cases, a single gene encoding one nuclear receptor is expressed in target tissues, along with co-activator and co-repressor proteins, although in some cases (e.g., human glucocorticoid receptor) there is extensive alternate splicing leading to multiple receptor isoforms. The liganded receptor binds to short target sequences called hormone response elements, which are arranged in direct or inverted repeats. These DNA-bound receptors recruit co-activators and/or co-repressors, and the complex interacts with the transcriptional machinery to regulate expression of the hormone-responsive genes. Some hormones have two receptors, such as estrogen receptors α and β, and many hormones elicit "non-genomic" signals, via extranuclear receptors that activate kinase cascades or open ion channels.

12.1.2 Enzymes of Steroidogenesis

Within each steroidogenic cell, a specific repertoire of enzymes catalyzes the necessary reactions to convert cholesterol into one or more final steroid products. The human genome contains six genes encoding steroidogenic cytochrome P450 enzymes. Some species of fish express more than one isoenzyme similar to a single human steroidogenic P450, each with a somewhat different spectrum of activities. Unlike most other biological processes involving P450s, steroidogenesis begins in the mitochondrion, and half of the steroidogenic P450s are type I enzymes, located in mitochondria, and half are type II enzymes, located in the endoplasmic reticulum (Table 12.2). As for all P450s, the steroidogenic P450s require a redox partner protein to receive electrons from reduced nicotinamide adenine dinucleotide phosphate (NADPH), which enables molecular oxygen binding and formation of the catalytically competent heme–oxygen complex.

Table 12.2 Classes of human steroidogenic enzymes and related proteins

I. Cytochromes P450
A. Type I (mitochondrial)
B. Type II (microsomal)
II. P450 Redox proteins
A. Flavoproteins
B. Iron-sulfur proteins
C. Hemoproteins
III. Oxidoreductase enzymes
A. Hydroxysteroid dehydrogenases
i. Short-chain dehydrogenase/reductases
ii. Aldo-keto reductases, including 5β-reductase
B. 5α-Reductases
C. Sugar phosphate dehydrogenases
IV. Conjugating/deconjugating enzymes
A. Sulfotransferases and sulfatases
B. Uridinediphosphate glucuronosyltransferases
C. Cosubstrate synthetases
V. Cholesterol mobilizing proteins
A. Steroidogenic acute regulatory protein
B. Translocator protein

The mitochondrial P450s receive electrons from the iron-sulfur protein ferredoxin (FDX1), which in turn accepts electrons from NADPH via the flavoprotein ferredoxin reductase (FDXR), whereas the microsomal P450s receive electrons from NADPH via P450-oxidoreductase (POR; reviewed in: [13]). Some hepatic P450s also catalyze some of the same reactions as the steroidogenic P450s, but with different rates and substrate specificities, as well as catabolic reactions with steroids. For example, hepatic P450 2C19 and 3A4 catalyze 21-hydroxylation of progesterone (but not 17-hydroxyprogesterone) with modest efficiency [14].

Another central class of steroidogenic enzymes is the hydroxysteroid dehydrogenases (HSDs). The HSDs primarily catalyze the terminal steps of steroidogenesis and regulate steroid metabolism in peripheral tissues and target organs. The HSD enzymes distribute into two structural classes, the short-chain oxidoreductase (SDR) and aldo-keto reductase (AKR) families. The SDR enzymes have β-α-β structures with a Rossman fold, and the AKR enzymes are β-barrel proteins. In general, two or more HSD enzymes

interconvert a hydroxysteroid and its cognate ketosteroid with a strong directional preference. In intact cells, one isoenzyme drives steroid flux in one direction and the other isoenzyme favors the reverse reaction. The HSD enzymes vary in the classes of steroids they metabolize, the carbon atom(s) where they perform chemistry, their affinity for various nicotinamide cofactors, and their directional preferences in intact cells [15, 16]. In particular, the 3β-hydroxysteroid dehydrogenase/$\Delta^5 \rightarrow \Delta^4$-isomerase (3βHSD) is required for the biosynthesis of all the major classes of steroid hormones, and 17β-hydroxysteroid dehydrogenase isozymes are required for the synthesis of androgens and estrogens.

Several types of enzymes form steroid conjugates, including sulfates, glucuronides, and esters. Several hydroxysteroids circulate in high abundance as sulfate conjugates with high protein binding, which slows their metabolism and clearance from plasma [17]. The steroid sulfotransferases (SULTs), steroid sulfatase, uridinediphosphate glucuronosyltransferases, and 5β-reductase are involved in reactions that promote protein binding, prevent metabolism, and enhance excretion. Certain cells acquire these steroid sulfates and remove the sulfate, yielding free steroids. Steroid sulfates are excreted in urine, but similar to xenobiotics, the majority of urinary steroids are excreted as glucuronide conjugates.

One crucial protein for steroidogenesis is the steroidogenic acute regulatory protein (StAR). StAR is not an enzyme but rather a short-lived protein that acts on the outer mitochondrial membrane [18] to enable the translocation of a specific cholesterol pool in the outer mitochondrial membrane to the inner mitochondrial membrane, where steroidogenesis begins [19]. StAR stimulates steroidogenesis about sevenfold above the StAR-independent rate [20]. The phosphorylation of StAR on Ser 195 in response to cAMP doubles its activity [21] in the gonads and in the ZF and ZR of the adrenal cortex. In the adrenal ZG, StAR activation derives mainly from increases in intracellular calcium [22]. Steroidogenesis is StAR-independent in the placenta and in organs that do not export significant amounts of steroids into the circulation, such as the brain. The translocator protein (TSPO) also participates in cholesterol mobilization, particularly with pharmacologic stimulation [23], but knockout of mouse TSPO shows it is not required for tropic hormone stimulation of steroidogenesis [24a].

The tissue patterns of expression of StAR, P450s, HSDs, and other enzymes and cofactors, direct the flux of precursor steroids to the final products. Each steroidogenic cell type yields a predominant steroid of a particular class that exerts a particular physiologic action. Enzymes in target tissues might convert a steroid to a more potent steroid hormone of the same class, a steroid hormone of another class, or an inactive steroid metabolite. The potential for peripheral conversion of steroids adds to the complexity of steroid biology and demonstrates how a single circulating hormone can exert diverse actions on the whole body based on the distribution of enzymes and receptors in various tissues. A summary of the major enzymes, redox partners, and other important proteins of human steroidogenesis are show in Table 12.3.

12.1.3 Pathways

The cleavage of cholesterol to the 21-carbon pregnenolone by P450scc is the first committed step in steroidogenesis, the rate-limiting step, and the site of chronic regulation. Only the adrenal cortex cells, the Leydig cells in the testis, the granulosa and less so theca cells of the ovary, and the trophoblast cells of the placenta synthesize enough pregnenolone from cholesterol to contribute to circulating steroid concentrations, although other cells produce enough pregnenolone to make autocrine steroid hormones. Given that pregnenolone is the common first intermediate, the types of steroids a specific cell produces depends on the repertoire of downstream enzymes present in that cell.

Tropic hormones, including adrenocorticotropin (ACTH) and angiotensin II in the adrenal and luteinizing hormone (LH) in the ovary and testis, signal via second messengers to drive acute rises in steroid production from many steroidogenic cells. StAR mediates this acute rise in steroido-

Table 12.3 Human steroidogenic enzymes and other proteins

	Gene	mRNA	Chromosome		Molecular
Protein	Size (kb)	Size (kb)	Locus	Exons	weight (kDa)
StAR	8	1.6	8p11.2	8	32
P450scc	30	2.0	15q23-q24	9	60
P450c11β	9.5	4.2	8q21-22	9	58
P450c11AS	9.5	4.2	8q21-22	9	58
P450c17	6.6	1.9	10q24.3	8	57
P450c21	3.4	2.0	6p 21.1	10	56
P450aro	130	1.5–4.5	15q21.1	10	58
3βHSD1	8	1.7	1p13.1	4	42
3βHSD2	8	1.7	1p13.1	4	42
11βHSD1	7	1.6	1q32-q41	6	32
11βHSD2	6.2	1.6	16q22	5	44
17βHSD1	3.3	1.4–2.4	17q11-q21	6	35
17βHSD2	63	1.5	16q24.1-q24.2	5	43
17βHSD3	67	1.2	9q22	11	35
17βHSD6	24.5	1.6	12q13	5	36
AKR1C1	14.3	1.2	10p14–p15	9	37
AKR1C2	13.8	1.3	10p14–p15	9	37
AKR1C3	13.0	1.2	10p14–p15	9	37
AKR1C4	22.1	1.2	10p14–p15	9	37
5α-Reductase 1	36	2.4 5p15	5p15	5	29
5α-Reductase 2	56	2.4	2p23	5	28
SULT2A1	17	2.0	19q13.3	6	34
PAPSS2	85	3.9	10q24	13	70
POR	69	2.5	7q11.2	16	77
FDX1	35	1.0–3.2	11q22	5	19
FDXR	11	2.0	17q24-q25	12	54
CYB5A	32	0.9	18q23	5	15
H6PDH	36.5	9.1	1p36	5	89
P4502R1	14.2	1.6	11p15.2	5	57
P450c27	40.5	2.4	2q35	9	57
P450c24	27.5	3.3	20q13	11	55
P450c1α	5	2.5	12q14.1	9	56

genesis by mobilizing cholesterol from a pool in the outer mitochondrial membrane and providing access to the cholesterol side chain cleavage enzyme (P450scc, CYP11A1) on the inner mitochondrial membrane. In the placenta, steroidogenesis is StAR-independent. The mechanism of StAR action is incompletely understood and the topic of recent reviews [25].

The human adrenal cortex contains three zones, each of which produces a single major steroid hormone product and various precursor and by-product steroids (Fig. 12.1). The human genome contains six genes encoding steroidogenic P450s, which are expressed in the three zones of the adrenal cortex, and in the testis, ovary, and placenta. Of these, the three genes in the CYP11 family encode mitochondrial P450s: P450scc (CYP11A1), 11β-hydroxylase (P450c11β, CYP11B1), and aldosterone synthase (P450c11AS, CYP11B2). The other three P450 enzymes are microsomal: 17-hydroxylase/17,20-lyase (P450c17, CYP17A1), 21-hydroxylase (P450c21, CYP21A2), and aromatase (P450aro, CYP19A1). The chemistry of each P450 enzyme is covered in detail in the next section.

The simplest pathway is progesterone (Prog) synthesis, which only requires P450scc and an SDR enzyme, 3βHSD. In the ZG of the adrenal

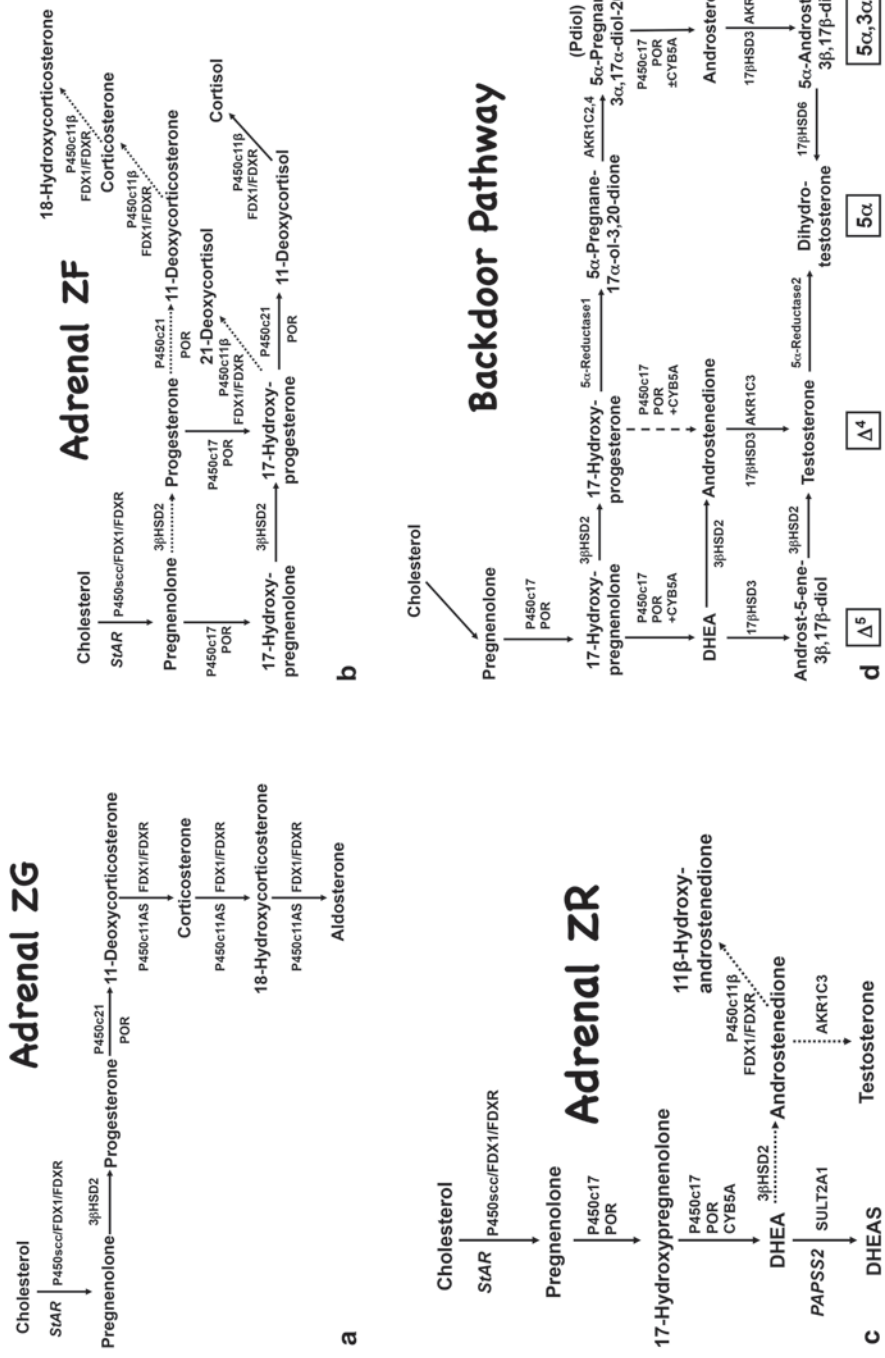

Fig. 12.1 Major steroidogenic pathways in the human adrenal gland. The ZG (**a**) produces aldosterone and a few intermediates using P450scc, P450c21, and P450c11AS. The ZF (**b**) contains P450scc, P450c17, P450c21, and P450c11β and primarily produces cortisol plus minor products, shown with dotted lines. These minor products and intermediates accumulate in the presence of enzyme deficiency or an enzyme inhibitor. The ZR (**c**) contains only P450scc and P450c17 but has abundant CYB5A, which limits efficient steroidogenesis to dehydroepiandrosterone (DHEA) and its sulfate (*DHEAS*) and smaller amounts of 19-carbon steroids such as testosterone. In the "backdoor pathway" (**d**), the 21-carbon steroids, primarily 17OHP, are 5α- and 3α-reduced to form 17OH-Allo prior to the 17,20-lyase reaction, ultimately yielding dihydrotestosterone without the intermediacy of dehydroepiandrosterone, androstenedione, or testosterone

cortex, the major product is the mineralocorticoid aldosterone, which regulates salt balance and thus extracellular fluid volume. In addition to P450scc and 3βHSD, the ZG contains P450c21, which converts Prog to 11-deoxycorticosterone (11DOC). Nascent 11DOC returns to the mitochondria, where P450c11AS performs the three sequential 11β-hydroxylase, 18-hydroxylase, and 18-oxidase reactions, oxidizing 11DOC to corticosterone, 18-hydroxycorticosterone, and finally aldosterone (Fig. 12.1a). The ZG has a single major pathway with no branch points and few additional products, mainly 18-hydroxycorticosterone. Unlike the other zones of the adrenal cortex, the major tropic stimulus is not a pituitary hormone but rather low serum potassium and angiotensin II, the latter produced when intravascular volume is low.

The adrenal ZF contains P450scc, 3βHSD, and P450c21 (as does the ZG) but lacks P450c11AS. Instead, the ZF expresses two additional steroid hydroxylases, P450c17 and P450c11β. Under adrenocorticotropin stimulation, these four P450 enzymes lead to the production of cortisol, which contains hydroxyl groups at carbons 11, 17, and 21 (Fig. 12.1b). The adrenal glands of rodents lack P450c17 and thus produce the dihydroxy steroid corticosterone instead. The K_m of human P450c17 is ~0.8–1 μM, whereas the K_m of 3βHSD is ~5.5 μM [26, 27], so that the human adrenal produces relatively little corticosterone. Thus, pregnenolone is preferentially converted to 17-hydroxypregnenolone (17OH-Preg), which accumulates and is converted to cortisol; the production of cortisol exceeds that of corticosterone by about 10:1. Both cortisol and corticosterone are glucocorticoids that regulate carbohydrate metabolism and response to stress. The ZG has some branch points and makes some additional products, such as 18-hydroxysteroids due to the low 18-hydroxylase activity of P450c11β [28]. Ordinarily, the precursors of cortisol are secreted in minimal quantities, but in congenital enzymatic defects collectively known as congenital adrenal hyperplasia (CAH), these steroids predictably accumulate. The diagnostic precursor steroids elevated in each condition are 11-deoxycortisol and 11DOC in 11-hydroxylase deficiency [29]; corticosterone and 11DOC in 17-hydroxylase deficiency [30]; and 21-deoxycortisol and 17-hydroxyprogesterone (17OHP) in 21-hydroxylase deficiency [31].

ACTH also stimulates steroidogenesis in the ZR. The major P450 enzyme downstream from pregnenolone in the ZR is P450c17. In contrast to the ZG, the ZR expresses low amounts of 3βHSD, which limits most steroidogenesis to the Δ^5-pathways, and expresses abundant cytochrome b_5 (CYB5A) [32, 33] and the sulfotransferase SULT2A1 [34]. CYB5A activates the 17,20-lyase activity of P450c17, yielding dehydroepiandrosterone (DHEA), which SULT2A1 converts to DHEA sulfate (DHEAS, Fig. 12.1c). The 17,20-lyase activity of human P450c17 is much more efficient in the Δ^5-pathway than the Δ^4-pathway [26, 35], which enhances DHEA synthesis in the ZR and limits the production of other 19-carbon steroids in this zone. Serine/threonine phosphorylation of P450c17, apparently by p38α (MAPK14), also selectively activates the 17,20-lyase activity in concert with CYB5A [36–38].

In pathologic states in which 17OHP accumulates, 19-carbon steroid production can follow an alternate or "backdoor pathway" if 5α-reductase is present [39], as in the neonatal tammar wallaby testis [40]. In this pathway, 17OHP undergoes 5α- and 3α-reduction to 5α-pregnane-3α,17α-diol-20-one (17-hydroxyallopregnanolone, 17OH-Allo, Fig. 12.1d). Human P450c17 catalyzes the 17,20-lyase reaction with 17OH-Allo to yield androsterone more efficiently than the normally dominant Δ^5-pathway, and CYB5A stimulates this reaction with 17OH-Allo only threefold [41]. Even a small amount of flux through the backdoor pathway is significant, because the products are potent androgens that are not substrates for P450aro. This pathway appears to participate in male sexual differentiation [42] and probably explains the virilization of 46,XX newborns with 21-hydroxylase deficiency [43] and possibly also the virilization of 46,XX newborns with POR deficiency due to certain mutations [44, 45].

Similar to the ZR, the testicular Leydig cells express P450c17 and CYB5A to optimize conversion of pregnenolone to DHEA under stimulation from LH. In contrast to the ZR, Leydig cells

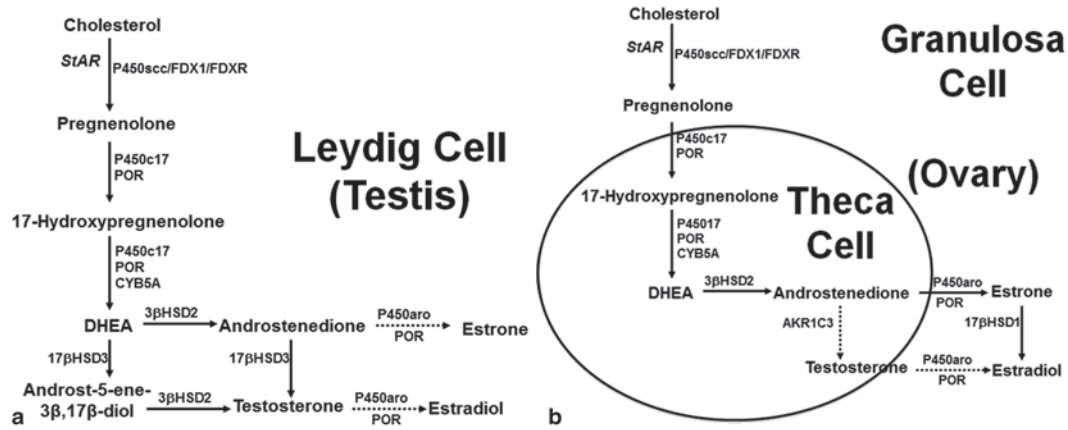

Fig. 12.2 Steroidogenic pathways in the human testis and ovary. Similar to the ZR of the adrenal cortex, the Leydig cell (**a**) expresses only two P450 enzymes, P450scc and P450c17, which enable testosterone production via redundant pathways. The ovary (**b**) uses two cell types to produce estrogens. The granulosa cells contain abundant P450scc and P450aro but little or no P450c17, so these cells generate pregnenolone and complete the conversion of androgens to estrogens (*outer area*). In contrast, the theca cells are deficient in P450scc and P450aro but express high amounts of P450c17, which catalyzes the conversion of pregnenolone to dehydroepiandrosterone (*DHEA; inner oval*)

lack SULT2A1 but express both 3βHSD and 17βHSD type 3, which complete the synthesis of testosterone (Fig. 12.2a).

In the ovary, steroidogenesis is more complex than in the testis, because two cell types participate, and the major product varies across the menstrual cycle. Follicle-stimulating hormone (FSH) drives pregnenolone synthesis in the granulosa cells, and LH activates the conversion of this pregnenolone to DHEA and then androstenedione in the theca cells via P450c17 and 3βHSD. The androstenedione returns to the granulosa cell, where P450aro (aromatase) and 17βHSD type 1 catalyze its aromatization to estrone and reduction to estradiol (Fig. 12.2b). After ovulation, the granulosa cells of that follicle transform to a corpus luteum, which produces Prog using only P450scc and 3βHSD (Fig. 12.3a).

In human pregnancy, Prog is initially produced from the corpus luteum of the ovary and subsequently from the placenta (Fig. 12.3b) at about 20 weeks of pregnancy, called the luteoplacental shift. Estrogens are produced by a complex system that involves both fetus and placenta (Fig. 12.3c). The 19-carbon steroid substrates for estrogen synthesis derive from the fetal adrenal, which primarily produces DHEAS via the same pathway as the ZR. The DHEAS is desulfated in the placenta and converted to androstenedione, estrone, and estradiol using the same pathway involving P450aro as in the ovary. In addition, P450 3A7 in the fetal liver converts much of the DHEAS to 16α-hydroxyDHEAS, which follows the same pathway as DHEAS to yield estriol. The human placenta produces estrone, estradiol and estriol in approximately a 15:5:80 ratio [46, 47]. Although estradiol is quantitatively minor, it is much more active and exerts the great majority of the estrogenic effect. Consequently, in human pregnancy, Prog is an exclusively placental product, while estrogen synthesis is a product of the feto-placental unit (Fig. 12.3).

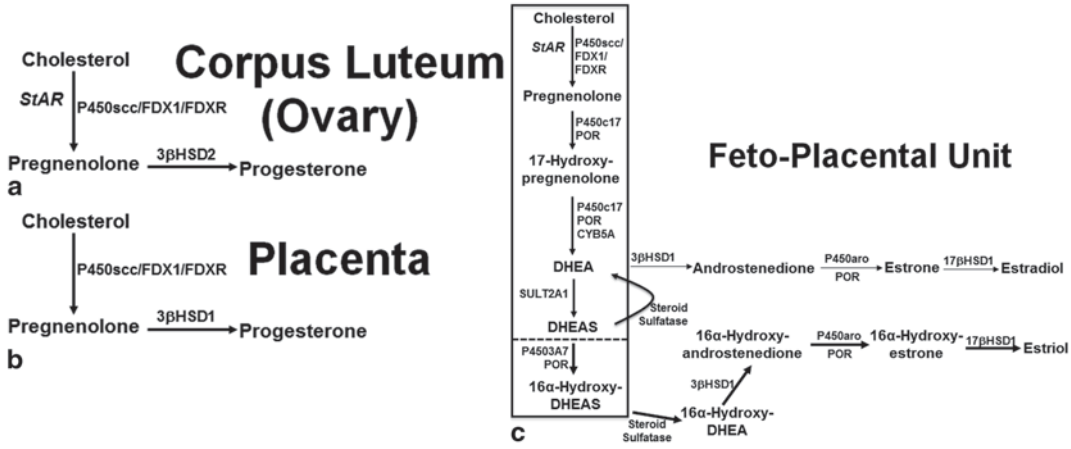

Fig. 12.3 Steroidogenesis in the human corpus luteum, placenta, and feto-placental unit. The only P450 in the corpus luteum (**a**) is P450scc, which limits steroidogenesis primarily to Prog. The placenta (**b**) contains the same pathway to Prog as the corpus luteum, except using 3βHSD type 1 rather than type 2, and the placenta lacks StAR. In addition, the fetoplacental unit (**c**) produces estrone, estradiol, and estriol. The fetal adrenal (*above dashed line in box*) is high in P450c17 and CYB5A, yielding dehydroepiandrosterone (*DHEA*), which enters the circulation as DHEA sulfate (*DHEAS*), a substrate for P450 3A7 in the fetal liver (*below dashed line in box*). In the placenta, steroid sulfatase removes the sulfate from 16α-hydroxyDHEAS, and 3βHSD1 oxidizes and isomerizes 16α-hydroxyDHEA, to yield 16α-hydroxyandrostenedione. Placental P450aro and 17βHSD1 catalyze the final transformations to 16α-hydroxyestrone and estriol, respectively.

12.2 Steroidogenic P450 Enzymes and Reactions

12.2.1 The Cholesterol Side-Chain Cleavage Enzyme (P450scc, CYP11A1)

P450scc is a mitochondrial P450 that receives electrons from NADPH via FDXR and then FDX1. The side-chain cleavage reaction is actually three consecutive oxygenation reactions, using one molecule of both NADPH and oxygen per cycle and yielding pregnenolone and isocaproaldehyde. The intermediates formed are first 22(*R*)-hydroxycholesterol and then 20(*R*),22(*R*)-dihydroxycholesterol. These intermediates are used as substrates experimentally because these hydroxysterols are more water-soluble than cholesterol and do not require StAR action to access P450scc in intact cells or mitochondria [20]. This multistep reaction is the rate-limiting step in steroidogenesis, with a turnover number of ~20 min^{-1} [48]. The k_{cat}/K_m ratios increase for each successive intermediate in the sequence of cholesterol oxidation, and the high K_D of pregnenolone (~3000 nM) favors product dissociation [48].

The X-ray crystal structure of P450scc in a complex with FDX1 and cholesterol demonstrates that the four-ring backbone of cholesterol binds at a 45° angle relative to the heme ring with the side chain extended over the heme [49]. This structure explains the regiochemistry of the hydroxylations and suggests that the hydroxycholesterol intermediates rarely dissociate before the subsequent reactions. P450scc also accepts other hydroxysterols as substrates for some or all of the reactions; it can 20- and 22-hydroxylate vitamin D and cleave the side-chain of 7-dehydrocholesterol [50–52].

Despite the complexity of the overall side-chain cleavage reaction, spectroscopic studies suggest that P450scc uses the canonical compound 1 (see Chaps. 3 and 4) for its reactions rather than a hydroperoxy-ferric heme intermediate, at least for the first hydroxylation of cholesterol [53]. The crystal structure of P450scc with bound 22-hydroxycholesterol shows an extensive network of ordered water molecules that

positions the substrate and supports proton transfer to the oxyferrous heme [54]. The 22-hydroxyl approaches to within <3 Å of the heme iron, but 22-hydroxycholesterol forms a less complete type 1 difference spectrum than cholesterol [54]. This result is consistent with greater mobility of the intermediate hydroxysterols than cholesterol in the active site pocket. Ketoconazole, as well as posaconazole, carbenoxolone, and selegiline, inhibits P450scc [55, 56], which contributes to its efficacy in treating Cushing syndrome (endogenous cortisol excess) and castration-resistant prostate cancer, the latter by further lowering testosterone production.

The interaction of FDX1 with P450 enzymes was first explored with P450scc. Residues 56–90 of FDX1 form an interaction domain, which includes the acidic residues D72, D76, D79, and E73. These negative charges comprise a surface that covers the Fe_2S_2 cluster and is critical for the interaction of FDX1 with positive surface charges of P450scc [57]. Overlapping sets of these negative charges on FDX1 drive interactions with positive surfaces of P450scc and FDXR [58, 59].

Deficiency of P450scc is a very rare disorder of steroidogenesis that abrogates synthesis of all steroids in the adrenal cortex and in the gonads. Since its first description in 2001 [60], as of mid-2014 only 19 cases have been reported [61]. Both complete and partial ("nonclassic") forms of P450scc deficiency have been described, in which the mutant enzymes retain 10–20 % of normal enzyme activity. P450scc deficiency closely resembles congenital lipoid adrenal hyperplasia (lipoid CAH), which results from mutations in the StAR protein. As StAR triggers cholesterol flux into mitochondria, its deficiency causes massive accumulation of cholesterol ester in enlarged adrenal glands; by contrast, in P450scc deficiency, the adrenals are not enlarged [62]. Lipoid CAH also occurs in a mild or nonclassic form, which preferentially impairs cortisol synthesis due to the greater quantity of cortisol normally produced compared to other active steroids [63].

12.2.2 Aldosterone Synthase (P450c11AS, CYP11B2)

A similar three-step one-enzyme process as for P450scc occurs in the biosynthesis of aldosterone. P450c11AS (CYP11B2, aldosterone synthase), which is expressed only in the ZG of the adrenal cortex, catalyzes one oxygenation at C-11 and two at C-18 in metabolizing 11DOC to aldosterone. The gene encoding P450c11AS is located on chromosome 8q21-22, 40 kb away from the gene for P450c11β (CYP11B1, steroid 11β-hydroxylase). These genes share 93 % sequence identity, but P450c11β (discussed below) is expressed only in the ZF of the adrenal cortex [63a]. The proximity of these genes and the similar activities of these enzymes explain the genetic origin of the disease glucocorticoid-remediable aldosteronism, also known as familial hyperaldosteronism type 1. A recombination event places a hybrid gene encoding an enzyme bearing aldosterone synthase activity downstream of an ACTH-responsive promoter, which drives aldosterone synthesis in the ZF and early onset hypertension [64, 65].

The X-ray crystal structure of human CYP11B2 in complex with 11DOC reveals that the steroid binds with the β-face in apposition to the catalytic surface of the heme, tethering the hydrogen atoms at C-11 and C-18 closest to the reactive iron–oxygen species [66]. This structure is consistent with the known catalytic activities of the enzyme. The 11β-hydroxylation reaction probably precedes the 18-hydroxylation due to the greater reactivity of the secondary carbon center over the 18-methyl group. Assays with the modified P450c11AS protein used for the X-ray structure show that both 11DOC and corticosterone are metabolized to aldosterone in vitro, following 3 or 2 oxygenations, respectively. Paradoxically, the most proximate intermediate to aldosterone, 18-hydroxycorticosterone, is very poorly metabolized to aldosterone, even though only one oxygenation is required. This result suggests that most of the aldosterone product derives from the population of 18-hydroxycorticosterone molecules that do not dissociate from the active

site prior to the final 18-oxidation reaction [66]. This in vitro result is consistent with the clinical observation that circulating concentrations of 18-hydroxycorticosterone are typically at least twofold higher than those of aldosterone.

Deficiency of P450c11AS is a very rare condition, which presents in infancy with salt wasting and low blood pressure. Most missense mutations in P450c11AS abrogate all activities, while a few others, such as R181W and V386A, preferentially impair the final 18-oxidase activity [67–69]. The identification of patients with selective loss of the 18-oxidase activity led to confusion that more than one enzyme was required to convert 11DOC to aldosterone, but the cloning and expression of the *CYP11B2* cDNA demonstrate that one enzyme performs all three P450-catalyzed reactions [70–72].

Excessive and autonomous aldosterone production, either from the ZG of both adrenal glands or from tumors of one adrenal gland, causes the condition primary aldosteronism, which accounts for 5–10% of human hypertension [73, 74]. Consequently, P450c11AS has been a target for drug development. Selective inhibitors of P450c11AS have been developed, with the major concern being to avoid simultaneous inhibition of P450c11β. Racemic fadrozole (4-(6,7-dihydro-5H-pyrrolo [1,2-c]imidazole-5-yl)-benzonitrile) was first studied as an aromatase inhibitor in the 1980s [75], but this compound also inhibits P450c11AS. The (*R*)-enantiomer is the more potent inhibitor (FAD286), with an IC_{50} in transfected cells of 6 nM [76, 77].

12.2.3 Steroid 11β-Hydroxylase (P450c11β, CYP11B1)

Both P450c11β and P450c11AS catalyze the 11β-hydroxylation of 11DOC to corticosterone, but the primary function of P450c11β is to complete the biosynthesis of cortisol from 11-deoxycortisol in the adrenal ZF. In addition, P450c11β has weak 18-oxygenase activity, converting corticosterone to 18-hydroxycorticosterone [78], but this enzyme cannot subsequently convert 18-hydroxycorticosterone to aldosterone. Human P450c11β shows broad substrate specificity and catalyzes 11β-hydroxylation of progesterone, 17OHP, androstenedione, and testosterone [79], and some of these products, such as 11β-hydroxytestosterone, retain (androgen) biological activity. Consequently, P450c11β appears to bind substrate similar to P450c11AS, yet no X-ray structure of P450c11β exists to explain these slight differences in the activities of the two enzymes.

Mutations in P450c11β cause a form of CAH, 11β-hydroxylase deficiency (11OHD). The clinical presentation of 11OHD derives from the accumulation of 11DOC, which is a mineralocorticoid, and shunting of cortisol precursors to androgens. Hence, girls are born with masculinized (ambiguous) external genitals from the androgen excess and later develop hypertension and low serum potassium from the 11DOC excess. Boys with 11OHD have normal male genitalia with the same blood pressure and electrolyte disturbances, and all patients with 11OHD are paradoxically prone to adrenal crisis with low blood pressure during significant illness due to glucocorticoid deficiency. In the Middle East, 11OHD is the second most common form of CAH, due to a founder mutation R448H found primarily in Jews of Moroccan ancestry [80], and G379V, found in Tunisia [81]. A mild or nonclassic form of 11OHD has been described in several patients, due to missense mutations that preserve 5–15% of wild-type enzyme activity [82].

The drug metyrapone (2-methyl-1,2-di(pyridin-3-yl)propan-1-one) has been used for decades to inhibit cortisol biosynthesis, primarily through its inhibition of P450c11β [83]. Metyrapone is a relatively weak inhibitor, requiring several grams per day in 3–4 divided oral doses. Etomidate (ethyl 1-(1-phenylethyl)-1H-imidazole-5-carboxylate), which is used as an anesthetic agent, is also a relatively weak inhibitor of P450c11β, but this off-target action can cause transient or sustained hypocortisolism [84, 85]. More recently, the compound LCI699 ((*R*)-4-(6,7-dihydro-5H-pyrrolo[1,2-c]imidazole-5-yl)-3-fluorobenzonitrile) has been studied as a potent P450c11β inhibitor. Originally developed as a P450c11AS inhibitor, participants in clinical trials had dose-

dependent lowering of cosyntropin-stimulated cortisol, consistent with P450c11β inhibition [86]. Subsequently, the drug was tested in Cushing's disease, and oral doses of 2–50 mg twice daily normalized cortisol production in a series of 12 patients [87]. Additional studies of metyrapone and LCI699 in the treatment of Cushing syndrome are underway.

12.2.4 Steroid 17-Hydroxylase/17,20-Lyase (P450c17, CYP17A1)

P450c17 is a 57 kDa microsomal P450, which receives electrons from POR rather than FDX1, unlike the three mitochondrial P450 enzymes discussed above. P450c17 is abundant the adrenal ZF and ZR, the Leydig cells of the testis, and the theca cells of the ovary. Small amounts of P450c17 are found in the human placenta [88], certain brain regions [89, 90] and other organs of the rat [91], and in prostate cancers [92, 93]; however, the significance of these findings remains under investigation. The adrenal ZF has minimal 17,20 lyase activity, hence the 17-hydroxylase activity leads to synthesis of the 21-carbon trihydroxysteroid cortisol, the major glucocorticoid in human beings and most other vertebrates. For P450c17 from most species, pregnenolone and Prog are comparably good substrates for the 17-hydroxylase reaction. With Prog as substrate, human P450c17 yields not only 17OHP but also 20–25% 16α-hydroxyprogesterone (16OHP) [94], and leucine substitution at A105, as is found in chimpanzee P450c17, reduces the 16OHP product to <10% [95]. Human P450c17 also 21-hydroxylates Prog, yielding 11DOC as ~1% of the products [96], and the enzyme 17-hydroxylates 5α-dihydroprogesterone (5α-pregnane-3,20-dione) and allopregnanolone (5α-pregnan-3α-ol-20-one) as well [41].

As with other P450-catalyzed hydroxylation reactions, C–H bond breaking appears to be the first chemical step. The product distribution with Prog reflects the stability of the carbon-centered radicals formed during turnover, in that the major product is 17OHP (C-17 forms a tertiary carbon radical), 16OHP is the next most abundant (C-16 forms a secondary carbon radical), and 11DOC is the minor product (C-21 forms a primary carbon radical). Pregnenolone, in contrast, forms only 17OH-Preg with no trace of alternate products, suggesting that the trajectories of these two substrates are quite different. Consistent with this mechanism, the product distribution for P450c17 with Prog substrate demonstrates large intramolecular (intrinsic) kinetic isotope effects (KIE) due to metabolic switching. For example, deuterium substitution at H-17 of progesterone shifts the product distribution to approximately 50% 16OHP, 45% 17OHP, and 5% 11DOC and yields a calculated intramolecular KIE of 4.1 [96]. Deuterium substitution at H-16α shifts product distribution to >90% 17OHP, and 33–40% of the 16OHP formed retains the deuterium, consistent with abstraction of H-16β and inversion of the carbon-centered radical prior to hydroxide radical recombination [96]. Whereas the C–H bond-breaking step contributes little to the overall rate of P450 enzymes with high turnover rates, this first chemical step is substantially rate limiting for the P450c17-catalyzed hydroxylation reactions. Studies with deuterium-labeled pregnenolone and Prog substrates yield intermolecular KIEs (^{D}V or $^{D}V/K$) averaging 2.0–2.5 with P450c17 wild type or mutation A105L [96].

The 17,20-lyase reaction, in contrast to typical P450-catalyzed hydroxylation reactions, involves the oxidative cleavage of a carbon–carbon bond. Several other P450 enzymes participating in steroid and sterol metabolism also catalyze reactions that break carbon–carbon bonds, including lanosterol demethylase (P450c51, CYP51A1), P450scc, and aromatase (P450aro). Human P450c17 catalyzes the 17,20-lyase cleavage of 17OH-Preg to DHEA 50–100 times more efficiently than 17OHP to androstenedione [26, 35]; however, the best substrate found thus far for the 17,20-lyase reaction is 17OH-Allo [41]. P450c17 from rodents favors 17OHP over 17OH-Preg for the 17,20-lyase reaction [97, 98], whereas the pig [8] and Xenopus [99] enzymes show high catalytic efficiency for both substrates. Some species of fish possess two genes encoding separate P450c17 isoenzymes, one with 17,20-lyase activity and the other without [100].

The mechanism of the 17,20-lyase reaction is not known, but the reaction requires NADPH and oxygen. The acyl fragment retains all the original atoms and incorporates one oxygen atom from O_2 during the final turnover [101]. This observation led to the proposal that the 17,20-lyase reaction proceeds through a ferric peroxide intermediate, which forms an adduct with the C-20 carbonyl before homolytic O–O bond cleavage and rearrangement. This mechanism predicts that hydrogen peroxide should substitute for NADPH and oxygen as co-substrate for the 17,20-lyase reaction, but neither hydrogen peroxide nor iodosobenzene supports catalysis for either the 17-hydroxylase or 17,20-lyase reactions for human P450c17, not even for a single turnover [102].

In contrast to other major sites of P450c17 expression, the 17,20-lyase activity in the adrenal ZF is low, limiting synthesis of 19-carbon steroids, which are precursors of androgens and estrogens. Among the reasons for this low 17,20-lyase activity is the paucity of CYB5A in the ZF compared to other cells expressing P450c17 [33, 34]. CYB5A stimulates the 17,20-lyase reaction with 17OH-Preg and 17OHP tenfold [26] but stimulates 17OH-Allo cleavage to androsterone only threefold [41]. The stimulatory action of CYB5A on the 17,20-lyase activity of P450c17 has been observed with microsomal enzyme preparations [26, 102] and with purified, reconstituted proteins [103]. The physiologic relevance of this in vitro phenomenon has been confirmed genetically with the description of patients having isolated 17,20-lyase deficiency due to mutations in the *CYB5A* gene [104, 105].

Apo-CYB5A lacking the heme moiety stimulates the 17,20-lyase of human P450c17 as well as holo-CYB5A, consistent with an allosteric effect [26], and redox-inactive Mn^{+2}-CYB5A stimulates 17,20-lyase activity [106]; however, scavenging of free heme in the reaction mixture has been suggested as an alternate explanation [107]. The CYB5A double mutation E48G + E49G stimulates 17,20-lyase activity < twofold yet retains normal electron transfer kinetics [108], suggesting that residues E48 and E49 form an allosteric interaction with P450c17. NMR studies confirm the participation of CYB5A residues E48 and E49 in interactions with P450c17 [109]. CYB5A residues D58 and D65 are essential for the stimulation of P450 2E1 and P450 2C19 activities, but are not required for stimulation of the 17,20-lyase activity of P450c17 [110].

CYB5A also influences the reactions of P450c17 with pregnenolone and allopregnanolone, whose products include a small amount of Δ^{16}- and 17α-hydroxy-19-carbon products in one step without 17-hydroxylated intermediates [41, 111, 112]. Acetic acid with one oxygen atom from O_2 is also formed during these reactions [101, 113, 114]. Human and pig P450c17 also catalyze these variants of the 17,20-lyase reaction with the 17β-carboxaldehyde analog of pregnenolone with similar product ratios [115]. P450c17 mutations (R347A, R347H, R358A, R358Q, and R449A) that impair 17,20-lyase activity with 17OH-Preg, however, retain normal CYB5A-stimulated activity with the 17β-carboxaldehyde substrate, forming the same alternate 19-carbon products [116]. In some species, these products are pheromones, the best characterized being androsta-5,16-diene-3β-ol and 5α-androst-16-en-3-one, which are components of boar taint [117].

P450c17 is encoded by the *CYP17A1* gene, which has a similar structure to the *CYP21A2* gene encoding the steroid 21-hydroxylase, P450c21 [118]. Mutations in *CYP17A1* cause a spectrum of disorders ranging from complete, combined 17-hydroxylase/17,20-lyase deficiency (17OHD) to partial deficiencies that variably impair these two main activities. The loss of 17,20-lyase activity eliminates androgen and estrogen synthesis, leading to sexual infantilism with female external genitalia, infertility, and pubertal failure regardless of chromosomal sex. Absence of 17-hydroxylase activity restricts adrenal steroidogenesis to the 17-deoxy pathway as in the rodent adrenal, which produces corticosterone as the major glucocorticoid. In 17OHD, circulating concentrations of corticosterone rise markedly, as do concentrations of its immediate precursor, 11DOC, to reach a new steady state. The profound 11DOC excess, however, activates the mineralocorticoid receptor and causes hypertension and potassium excretion.

Some missense mutations in P450c17, including R347H, R347C, R358Q [119, 120], and E305G [121] or G539R in POR [122] minimally disrupt 17-hydroxylase activity, but markedly impair 17,20-lyase activity and clinically cause isolated 17,20-lyase deficiency (ILD). Boys with ILD have incomplete masculinization of the external genitals with low testosterone, pubertal failure, and infertility, but lack the hypertension and hypokalemia of 17OHD. Girls with ILD show failure of puberty and adrenarche like boys with ILD [123]. These mutations causing ILD demonstrates that the 17,20-lyase activity of P450c17 is more sensitive to conditions and more vulnerable to disruption than the 17-hydroxylase activity. The 17,20-lyase activity of purified P450c17 is very sensitive to phospholipid composition in reconstituted assays, favoring anionic head groups such as phosphatidylinositol and phosphatidylserine over the cationic phosphatidylcholine [110]. P450c17 phosphorylation also stimulates 17,20-lyase activity [36, 37, 124]. Mitogen-associated protein kinase-14 (MAPK14, p38α) is the most active kinase thus far identified [38], and protein phosphatase 2A (PP2A) reverses the activation via P450c17 dephosphorylation [124].

Based on the absence of androgen production in patients with 17OHD, P450c17 has been a target for drug design to treat androgen-dependent disorders, primarily prostate cancer [125]. Abiraterone acetate, which is a prodrug for abiraterone, a potent and selective P450c17 inhibitor, improves survival of patients with castration-resistant prostate cancer (CRPC) after [126] or before taxane chemotherapy [127]. Abiraterone acetate is FDA-approved for the treatment of CRPC in combination with prednisone, which lowers pituitary ACTH production and thus prevents the accumulation of 11DOC and the development of hypertension and hypokalemia as seen in 17OHD. Abiraterone contains a 3-pyridyl ring attached to an unsaturated D-ring of the DHEA nucleus, which binds tightly to the heme iron with a spectral (K_S) affinity constant of <3 nM [128]. Other P450c17 inhibitors are under clinical development, such as galeterone (TOK-001), which differs from abiraterone in that a benzimidazole moiety replaces the 3-pyridyl ring [129]; orteronel (TAK-700), which is a nonsteroidal inhibitor [130]; and VT-464, for which preliminary evidence suggests preferential inhibition of 17,20-lyase activity over 17-hydroxylase activity [131].

The structure of human P450c17 has been modeled with and without bound substrates [102], and X-ray structures with bound abiraterone or galeterone have been solved [132]. The model predicts that the substrate binds with the steroid nucleus parallel to the heme ring, with the α-surface of the D-ring nearest the heme iron [102]. The X-ray structures show the heterocyle nitrogen of the inhibitors tightly bound to the iron of the heme and the 3β-face of the steroid nucleus forming hydrophobic interactions with the I-helix. A pregnenolone molecule can be modeled into the space that abiraterone occupies in the structure, and this orientation places the H-17 atom in close proximity to the heme iron [132]. The 3β-hydroxyl group of abiraterone forms a hydrogen bond with the side-chain oxygen of N202, and ordered water molecules contribute to a larger hydrogen-bonding network, which also includes E305, R239, and Y201. Abiraterone analogs with different A-B ring structures (3-keto-Δ^4; 5α,3α-hydroxy; 5α,3-keto; and 3α-hydroxy-Δ^4), however, are also potent inhibitors of human P450c17 and form type 2 difference spectra with <1 nM affinities [128]. Consequently, the active site residues that interact with the A-ring oxygen appear to be capable of significant reorganization in order to accommodate significant structural variation in the ligand. Abiraterone and these analogs all show mixed inhibition patterns, suggesting a second inhibitory binding site for these compounds as well [128].

12.2.5 Steroid 21-Hydroxylase (P450c21, CYP21A2)

P450c21 shares 39% amino acid identity with P450c17, as well as similar gene structures and common substrates. In contrast, the chemistry of the reactions these two enzymes catalyze and their functions in human physiology are quite different. The only known substrates for P450c21 are Prog, 5α-dihydroprogesterone (5α-pregnane-

3,20-dione), and 17OHP—the best substrate and the ligand that affords the strongest type 1 spectral change [133]. For all of these substrates, P450c21 hydroxylates the electron-deficient 21-methyl group, and with Prog, human P450c21 also forms a trace of 16OHP. Residue V359 appears to be critical for restricting substrate trajectories, which limits hydroxylation to the relatively unreactive C-21 methyl group. Site-directed mutations that progressively reduce the bulk of V359 (V359A, V359G) increase the fraction of 16OHP formed to 40% and 90%, respectively [134]. As is true for P450c17, metabolic switching occurs with deuteration at C-21 or C-16, with intramolecular KIE values of 2.5–6.2 for wild-type P450c21 and for the V359A mutant [96]. C–H bond breaking is partially rate determining for P450c21, with intramolecular KIE values of 1.9–3.8 [96]. The common Δ^5-steroids are not substrates and are poor inhibitors.

The X-ray crystal structure of bovine P450c21 contains two molecules of 17OHP bound with high occupancy [135]. A 17OHP molecule in the active site is positioned with the steroid nucleus perpendicular to the heme ring with the 21-methyl group suspended in close proximity to iron–oxygen complex and the A-ring distal to the heme. In this orientation, the more reactive hydrogen atoms are too far away to undergo hydroxylation for the wild-type enzyme, which restricts chemistry to 21-hydroxylation. The second molecule of 17OHP is bound too far from the heme to undergo hydroxylation, and this ligand might serve an allosteric or structural function or might be an artifact of the crystallization conditions. The structure of P450c21 with bound Prog has not been reported. Because a clinical utility has not been proposed, few efforts have been devoted to the development of P450c21 inhibitors, although some halogenated steroids and ketoconazole are weak substrates and inhibitors, respectively [136].

The *CYP21A2* gene, which encodes P450c21, resides in a duplicated region within the human leukocyte antigen (HLA) locus on chromosome 6p21. The duplication contains the *CYP21A1P* pseudogene [137–139], which differs from the *CYP21A2* gene in ten areas, most of which are deleterious single base pair substitutions or deletions. Genetic recombination events in this region are common, including large or partial deletions, as well as gene conversion events that incorporate one or more segments of the *CYP21A1P* pseudogene into the *CYP21A2* gene. When both copies of the *CYP21A2* gene are mutated, most often from deletion or pseudogene conversion, 21-hydroxylase deficiency (21OHD) results, by far the most common form of CAH. Severe or classic 21OHD occurs in 1:16,000 live births worldwide [140], while a partial or nonclassic form occurs in 1:1000 individuals and up to 1:27 in certain populations [141].

In classic 21OHD, severe deficiency of cortisol and often aldosterone can lead to low blood pressure and cardiovascular collapse in infancy if untreated. These individuals are prone to similar adrenal insufficiency crises throughout life during systemic illness, such as infection or hemorrhage. Simultaneously, the block in 21-hydroxylation causes large amounts of precursor steroids to accumulate, and these intermediates follow the only pathways remaining, primarily to androgens. The androgen excess during fetal life causes various degrees of external genitalia virilization in girls, a disorder of sex development (46,XX DSD). Androgen excess persists throughout life, requiring treatment with glucocorticoids to lower ACTH and to prevent precursor steroids from accumulating. Nonclassic 21OHD results from mutations that preserve <20% of enzyme activity, primarily mutation V281L [142]. Cortisol production is preserved in nonclassic 21OHD, but at the expense of moderate precursor accumulation and resultant androgen excess. Most patients diagnosed with nonclassic 21OHD are either girls, who experience early development of pubic hair or accelerated growth, or young women, who present with irregular menstrual periods, unwanted facial and body hair, acne, and subfertility. Boys are rarely diagnosed with nonclassic 21OHD, most commonly due to early development and rapid progression of secondary sexual characteristics.

12.2.6 Aromatase (P450aro, CYP19A1)

The aromatase (P450aro) enzyme is so named because this enzyme catalyzes the conversion of 19-carbon androgens to 18-carbon estrogens, which contain an aromatic A-ring. Similarly to P450scc and P450c11AS, P450aro performs three cycles of oxygenation that remove the C-19 methyl group as formic acid. The first two oxygenations occur at C-19 itself, affording the 19-hydroxy and 19-oxo-intermediates, the latter via dehydration of the transient 19-*gem*-diol. Nearly all of the early studies used human placental microsomes as the source of enzyme. Isotopic labeling studies [143] have shown that the 19-methyl group is removed as formic acid (HCOOH) and that the 1β-hydrogen is lost to water [144] with retention of the 1α-hydrogen [145]. With androstenedione, the 2β-hydrogen is preferentially lost, whereas for testosterone, there is no stereochemical preference for enolization [146]. The first and third oxygen atoms incorporated into substrate are retained in the formic acid product [147], and active-site residues direct loss of the second incorporated oxygen from the 19-*gem*-diol [148]. The 3-oxygen of the substrate is retained, excluding Schiff base formation between an enzymic lysine and C-3 during catalysis [149].

The mechanism of the last oxygenation and the A-ring aromatization remains controversial. If loss of formic acid from C-19 and the 1β-hydrogen occurs early in this process, a second double bond would be introduced in the A-ring, and tautomerization of the 3-ketone would complete the aromatization reaction. Other proposals include a mechanism in which a ferric peroxide attacks the C-19 oxo-intermediate [147] and oxygen insertion at H-2β, forming the C-19 oxo, 2β-hydroxy-intermediate. Consistent with the latter model, 2β-hydroxy-19-oxoandrostenedione decomposes nonenzymatically to estrone with elimination of the 1β-hydrogen atom [145, 150].

The 19-hydroxy and 19-oxo intermediates readily dissociate from the active site before subsequent rounds of turnover in what is known as a distributive multistep enzyme process [144]. Pre-steady-state kinetics with purified, recombinant human P450aro demonstrate that androstenedione binds more tightly than its 19-hydroxy, 19-oxo, or estrone products (K_d=0.13 vs. 1.5–4.0 μM) and that its turnover is slower than for the two subsequent intermediates (k_{cat}=0.06 vs. 0.13–0.42 s^{-1}) [151]. Single-turnover and pulse-chase experiments corroborate earlier studies showing a distributive mechanism with release of intermediates [151].

Earlier studies suggested that the third oxygenation occurs either at C-2 or C-10, but these mechanisms do not explain the incorporation of the third oxygen atom into formic acid product. Currently, two mechanisms remain consistent with the experimental data, and neither can be excluded [152]. In the first, a nucleophilic attack of the ferric peroxide on the 19-aldehyde forms a tetrahedral intermediate, followed by homolytic cleavage of the peroxide bond, leaving unpaired electrons both on the steroid and the iron–oxygen intermediate. Homolytic or heterolytic abstraction of H-1β occurs, and rearrangement follows, with release of formic acid. One study using molecular dynamics simulation and hybrid quantum mechanics/molecular mechanics favors this mechanism [153]. In the second mechanism, compound 1 (see Chaps. 3 and 4) of the enzyme sequentially removes hydrogen atoms H-1β and one O–H from the 19-*gem*-diol, yielding the diradical intermediate, which fragments as in the alternative mechanism. This mechanism is consistent with results from studies with 19-substituted substrates [154] and density function theory calculations [155].

The estrogen dependence of most breast cancers has spurred interest in developing aromatase inhibitors. Early compounds such as 10-propargylestr-4-ene-3,17-dione [156], 4-hydroxyandrostenedione [157], and 6-ketoandrostenedione [158], all steroidal compounds, were primarily mechanism-based inactivators. Subsequently, azole-based nonsteroidal aromatase inhibitors including fadrozole [75], letrozole [159], and anastrozole [160] were developed, and the latter two compounds are now first-line therapy for estrogen receptor-positive metastatic breast cancer in postmenopausal women [161].

The X-ray crystal structure of human P450aro was the first structure solved for a steroidogenic P450 [162]. In this structure, androstenedione fits tightly in the pocket above the heme ring, with the β-face of the steroid and the C-19 methyl group closest to the heme iron, which is consistent with the known chemistry of the enzyme. The position of the steroid substrate resembles that of 11DOC in the P450c11AS structure, with the steroid moved to place the A-ring 3–4 Å closer to the heme iron [66]. This similarity explains how fadrozole inhibits both enzymes, but the P450aro structure does not explain the tight and selective binding of the fourth-generation aromatase inhibitors anastrozole and letrozole. If the steroid is removed from the structure of the P450aro- androstenedione complex, these two inhibitors do not fit in the vacated pocket, which suggests that the enzyme undergoes substantial conformational changes upon inhibitor binding to a very flexible active site [162].

P450aro is widely expressed in biologically significant amounts in many cells and tissues such as brain, bone, breast, and fat [163]. The 130 kb *CYP19A1* gene contains at least five distinct promoters that direct its expression in the placenta and ovary, which are the major sites of estrogen synthesis, as well as in extraglandular tissues. In each cell type, the distinct promoters function to provide the regulation of enzyme expression characteristic of that tissue. The extraglandular aromatization of androgens is a prime example of local enzyme-mediated or pre-receptor regulation of hormone action. Many behavioral effects of androgens, for example, are mediated by conversion to estrogen in the brain [164].

12.2.7 Catabolic P450-Mediated Steroid Metabolism

Additional hepatic metabolism of steroids contributes to their inactivation and also to extra-adrenal conversion to active steroids. Both CYP3A4 and CYP2C19 are progesterone 21-hydroxylases that yield 11DOC as a minor product along with other hydroxysteroids [14]. CYP3A4 catalyzes 6β-hydroxylation of progesterone, cortisol, and testosterone; this reaction accounts for a considerable amount of hormone inactivation, particularly when it is orally administered. This phenomenon is important, because patients taking CYP3A4 inducers and inhibitors while receiving cortisol replacement might require dose adjustment. The most profound effect is observed with adrenocortical cancer patients receiving mitotane therapy. Mitotane potently induces CYP3A4 expression and leads to more than a tenfold increase in 6β-hydroxycortisol production [165], which mandates hydrocortisone dose increases in these patients.

Another clinically important drug–drug interaction involving P450 3A4 occurs in patients taking the homeopathic herbal supplement, Saint John's Wort. The extract of this plant contains the compound hyperforin, which binds to and activates the human pregnane X receptor (PXR), thus inducing P450 3A4 expression in the liver and increasing the rate of metabolism for many drugs, including steroids [166, 167]. In addition to endogenous steroids, the consumption of Saint- John's Wort increases the metabolic rate of exogenous steroid drugs, particularly low-dose oral contraceptives with narrow therapeutic windows [168, 169].

12.3 P450 Enzymes in Vitamin D Synthesis

Vitamin D is a secosteroid derived from 7-dehydrocholesterol. The final step in the biosynthesis of cholesterol is the conversion of 7-dehydrocholesterol to cholesterol by 7-hydroxycholesterol reductase (3β-hydroxysterol Δ^7-reductase, DHCR7), the enzyme that is disordered in Smith-Lemli-Opitz syndrome (OMIM 270400) [170]. Patients with SLO, DHCR7-null mice, and animals given inhibitors of DHCR7 have augmented vitamin D synthesis [171]. Ultraviolet radiation at 270–290 nm directly cleaves the 9–10 carbon–carbon bond of the cholesterol B ring in human skin, converting 7-dehydrocholesterol to cholecalciferol (vitamin D_3, Fig. 12.4) [172]. Plants produce a closely related sterol, ergocalciferol (vitamin D_2), that has nearly the same properties

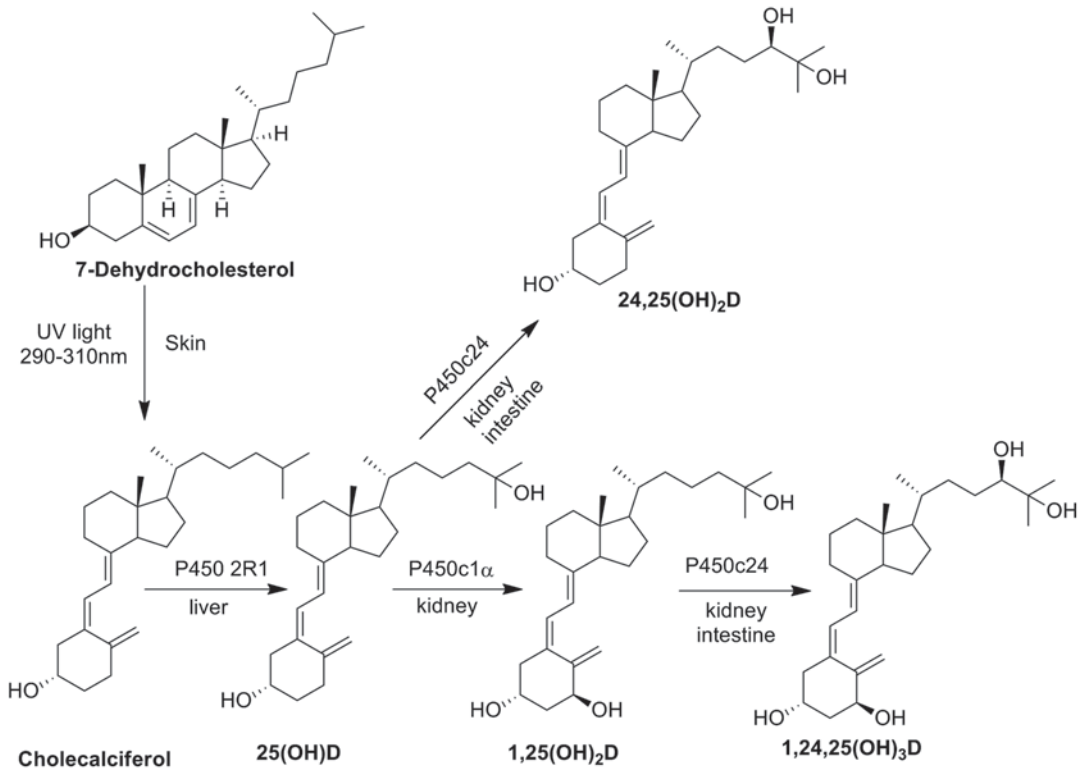

Fig. 12.4 Biosynthesis of major vitamin D metabolites. In skin, 7-dehydrocholesterol undergoes light-induced rearrangement to vitamin D_3 (cholecalciferol), via sequential retro-Diels–Alder reaction and [1,7]-sigmatropic shift. P450 2R1 and probably other enzymes catalyze vitamin D 25-hydroxylation to 25OHD, which is bound to circulating proteins in large amounts. P450c1α converts 25OHD to $1,25(OH)_2D$ (calcitriol), the active form of vitamin D. P450c24 catalyzes the inactivation of both 25OHD and $1,25(OH)_2D$ to $24,25(OH)_2D$ and $1,24,25(OH)_3D$, respectively

as cholecalciferol. Both forms of vitamin D are biologically inactive pro-hormones that are then activated, and subsequently inactivated, by the same P450 enzymes. This section will refer to "vitamin D," meaning both D_2 and/or D_3.

12.3.1 Vitamin D 25-Hydroxylases

Several hepatic P450s catalyze the 25-hydroxylation of vitamin D to 25-hydroxyvitamin D (25OHD). Physiologic regulation of this 25-hydroxylation has not been demonstrated, and circulating concentrations of 25OHD are primarily determined by dietary intake of vitamin D and exposure to sunlight. 25OHD is the most abundant form of vitamin D in the blood, but is a biologically inactive pro-hormone, having minimal capacity to bind to the vitamin D receptor. Cell fractionation studies found 25-hydroxylase activity in both mitochondria and microsomal fractions. Screening of rat liver cDNA expression libraries with antisera to a purified rat liver 25-hydroxylase preparation yielded the cDNA for an enzyme then called P450c25 [173, 174]. This enzyme, now known as mitochondrial P450c27 or CYP27A1, can also hydroxylate carbons 26 and 27 to initiate bile acid synthesis [175]. The subsequent cloning of the mitochondrial 1α- and 24-hydroxylases showed that CYP27A1 was structurally related, suggesting that CYP27A1 might be a major vitamin D 25-hydroxylase; however, patients with CYP27A1 mutations have a lipid disorder (cerebrotendinous xantho-

matosis) without a disorder in calcium metabolism [175, 176], suggesting that at least one other enzyme besides CYP27A1 was also a vitamin D 25-hydroxylase.

Microsomal P450 2R1 (CYP2R1) is a vitamin D 25-hydroxylase that has a higher affinity for vitamin D than CYP27A1 [177]. P450 2R1 is highly specific for vitamin D 25-hydroxylation. A homozygous P450 2R1 mutation L99P was then found in two unusual Nigerian patients, and this mutation dramatically reduced 25-hydroxylase activity in vitro [178]. However, through the middle of 2014, no further cases of *CYP2R1* mutations or other causes of 25-hydroxylase deficiency have been reported, indicating that 25-hydroxylase deficiency is exquisitely rare, and suggesting that both P450 2R1 and other enzyme(s) (possibly CYP27A1), are effective 25-hydroxylases in vivo, so that symptomatic disease is only seen when there is a P450 2R1 mutation in the presence of another stressor such as neonatal hypoparathyroidism or nutritional vitamin D deficiency. P450 2R1 is widely expressed, possibly accounting for the persistent vitamin D 25-hydroxylation in patients with liver failure [179].

The crystal structure of P450 2R1 with vitamin D_3 bound in its catalytic site shows a typical microsomal cytochrome P450 structure, but with a more closed, tight conformation and with hydrophobic residues lining the substrate-binding pocket, so that the geometry is only suited to binding planar hydrophobic molecules such as sterols [180]. The L99 residue is located in the B-helix, near to, but not directly involved in binding vitamin D. The substrate adopts the open or extended conformation as drawn in Fig. 12.4, with the side-chain containing C-25 hovering above the heme iron. The A-ring projects towards the surface of the protein and forms a hydrogen bond network between the 3β-hydroxyl group, a water molecule, and residues in the F- and G-helices [180].

12.3.2 Vitamin D 1α-Hydroxylase

The active form of vitamin D hormone, 1α,25-dihydroxyvitamin D ($1,25(OH)_2D$ or calcitriol), is produced by the 1α-hydroxylation of 25OHD. A mitochondrial 1α-hydroxylase variously termed 25-hydroxyvitamin D-1α-hydroxylase, P450c1α, or CYP27B1 catalyzes this critical reaction. Circulating $1,25(OH)_2D$ primarily derives from its synthesis in the kidney, but 1α-hydroxylase activity also is found in keratinocytes, macrophages, osteoblasts and placenta [181–183]. The rate-limiting step in the bioactivation of vitamin D is 1α-hydroxylation, and the renal enzyme activity is tightly regulated by parathyroid hormone (PTH), calcium, phosphorus, and $1,25(OH)_2D$ itself. Due to the low abundance of this protein in renal mitochondria, immunologic approaches could not be used to clone the 1α-hydroxylase, as had been done for the 24- and 25-hydroxylases. However, in the second half of 1997, four independent groups using different approaches reported the cloning of the human, rat, and mouse vitamin D-1α-hydroxylase cDNAs [184–188], and the human gene [185, 189], subsequently termed *CYP27B1*. One group used mice with a knocked-out vitamin D receptor to induce overproduction of 1α-hydroxylase, then screened a cDNA expression library for activation of a vitamin D receptor construct [188]. Two other groups enriched renal 1α-hydroxylase mRNA by feeding rats a diet low in calcium and phosphorus, then used probes complementary to the conserved P450 heme-binding site to identify candidate sequences [186, 187].

The first human clone was obtained by using RNA from primary cultures of human keratinocytes, which have substantial 1α-hydroxylase activity when grown in low-calcium medium [190], and screening cDNA with oligonucleotides corresponding to the conserved sequences of the ferredoxin-binding sites and heme-binding sites of other P450s [184]. The human *CYP27B1* gene on 12q14.1 is only 5 kb in length, is single copy, and comprises nine exons and eight introns [189]. Although it is substantially smaller than the genes for other mitochondrial P450 enzymes, its intron/exon organization is very similar, particularly to that of P450scc. This finding strongly suggests that although the mitochondrial P450 enzymes retain only 30–40% amino acid sequence identity with each other, they all belong to a single evolutionary lineage. P450c1α catalyzes con-

version of 25OHD to $1,25(OH)_2D$, with a K_m of 2.7×10^{-7} M, close to circulating concentration of 25OHD.

Deficient 1α-hydroxylase activity, characterized by the infantile onset of severe hypocalcemia, moderate hypophosphatemia, and responsiveness to physiologic doses of calcitriol, has been called "hereditary pseudo-vitamin D deficiency rickets" (PDDR), "vitamin D dependency" because of its responsiveness to active vitamin D, or "vitamin D-dependent rickets type I." This disease is now more simply and appropriately termed "vitamin D 1α-hydroxylase deficiency." Affected persons are normal at birth but have growth retardation, poor motor development, and generalized muscle weakness by 2 years of age. Affected children develop hypocalcemia, hypophosphatemia, increased serum alkaline phosphatase activity, and increased serum PTH; some develop hypocalcemic seizures. Serum concentrations of $1,25(OH)_2D$ are low despite normal concentrations of 25OHD; responses to administration of $1,25(OH)_2D$ are excellent.

Vitamin D 1α-hydroxylase deficiency is rare in most populations, but may be common in isolated populations due to founder effects. Among French Canadians in the Charlevoix-Saguenay-Lac Saint Jean area of Quebec, the carrier rate is 1/26, so that the incidence of affected individuals is 1 in 2700 in this population [191]. This high incidence in French Canadian families permitted genetic mapping of vitamin D 1α-hydroxylase deficiency to chromosome 12q14 [192]. Since the first description of a mutation in the *CYP27B1* gene [184], over 100 genetically confirmed cases of vitamin D 1α-hydroxylase deficiency, involving at least 38 different mutations in the *CYP27B1* gene, have been reported [193–200].

Although vitamin D 1α-hydroxylase deficiency is rare, an early study identified P450c1α mutations in 19 patients from 17 families of multiple ethnicities [195]. Microsatellite haplotyping and DNA sequencing showed that French-Canadian patients from the Charlevoix region all carried a single haplotype and the same frameshift mutation. This study also found a 7 bp duplication on seven alleles in six families, but this mutation was associated with several different microsatellite haplotypes and was found in several unrelated ethnic groups, indicating that this mutation has arisen de novo several times [195]. Among the 14 mutations identified in this study, none had measureable activity in vitro. A few P450c1α mutations have been described that retain partial activity and cause mild disease. The mutation E189G retained 22% of normal activity in vitro and caused mild disease, while L343F retained 2.3% of wild type activity [200], and the mutation G102E retained about 20% of normal activity [197]. Nevertheless, there is considerable phenotypic variation among patients with vitamin D 1α-hydroxylase deficiency who have mutations lacking assayable activity; the basis of this poor correlation of the clinical findings with the activities of the mutant P450c1α enzymes in vitro remains unclear.

12.3.3 Vitamin D 24-Hydroxylase

Calcitriol may be inactivated by P450 3A4 in liver, but one of the more important mechanisms for vitamin D inactivation is via its 24-hydroxylation by P450c24 (CYP24A1). This mitochondrial enzyme can catalyze the 24-hydroxylation of 25OHD to $24,25(OH)_2D$ and of $1,25(OH)_2D$ to $1,24,25(OH)_3D$, primarily in the kidney and intestine [201, 202]. Both reactions initiate inactivation of vitamin D, although some evidence suggests some activities for these 24-hydroxylated compounds. P450c24 was cloned by purifying the protein from rat renal mitochondria, raising a polyclonal antiserum, and screening a rat kidney cDNA expression library [202]. The human cDNA [201] and gene [203] were cloned soon thereafter. P450c24 is induced by $1,25(OH)_2D$, thus favoring its inactivation by 24-hydroxylation as a mechanism to regulate the amount of available $1,25(OH)_2D$ [204].

Deficient P450c24 activity is a recently described cause of neonatal hypercalcemia, with hypercalciuria or nephrocalcinosis, normal 25OHD levels, normal to moderately elevated $1,25(OH)_2D$ levels, and low PTH [205]. Another infant had failure to thrive, hypercalcemia, hypercalciuria, bilateral nephrocalcinosis, suppressed

PTH, undetectable PTH-related protein, and normal 25OHD and 1,25$(OH)_2$D [206]. The loss-of-function mutations found in these patients lacked P450c24 activity in vitro. *CYP24A1* splicing mutations were found in an adult with nephrocalcinosis, hypercalcemia, hypercalciuria, elevated 1,25$(OH)_2$D, and undetectable 24,25$(OH)_2$D [207]. Thus, *CYP24A1* mutations can cause severe neonatal hypercalcemia, and milder mutations that retain partial activity can cause hypercalcuria and nephrocalcinosis in adults.

The structure of rat P450c24 at 2.5 Å resolution shows an open cleft flanked by conserved hydrophobic residues on helices A′ and G′, with a membrane-directed substrate-access channel, stabilized by conserved aromatic residues on helices B′, F and G, leading to the heme. Docking of 1,25$(OH)_2$D shows that the hydrophobic substrate fits well in this channel. The proximal surface of P450c24 contains basic residues from the K, K″, and L helices and an adjacent lysine-rich loop that define the adrenodoxin binding site [208]. This structure is remarkably similar to that of human P450scc [49, 54] despite the low amino acid sequence identity.

the structures of substrate-bound human CYP17A1 mutation A105L were recently published:

Petrunak EM, DeVore NM, Porubsky PR, Scott EE (2014) Structures of Human Steroidogenic Cytochrome P450 17A1 with Substrates. J Biol Chem 289:32952-32964.

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P450 Enzymes in Lipid Oxidation

13

Matthew L. Edin, Jennifer Cheng, Artiom Gruzdev, Samantha L. Hoopes and Darryl C. Zeldin

13.1 Introduction

13.1.1 Arachidonic AcidMetabolizing Enzymes

While the cytochrome P450 (CYP) superfamily is an extensively studied enzyme system involved in xenobiotic metabolism, it was only more recently identified as a significant "third pathway" of arachidonic acid (AA) metabolism. In the first pathway, cyclooxygenases (COXs) metabolize AA to prostaglandin H_2 (PGH_2). Subsequently, various synthases convert PGH_2 to prostaglandins (PGs), thromboxane A_2 (TXA_2) and prostacyclin (PGI_2). The TXA_2 and PGI_2 synthases belong to the CYP superfamily. In the second pathway, lipoxygenase (LOX) enzymes convert AA to labile hydroperoxy intermediates that go on to form the leukotrienes, hydroxyeicosatetraenoic acids (HETEs), and lipoxins. COX and LOX metabolism of AA has been extensively studied and their eicosanoid products play important functional roles in a wide array of biological processes including inflammation, cellular proliferation, and intracellular signaling [1, 2]. Multiple subfamilies of CYP enzymes metabolize AA to three types of eicosanoid products (Fig. 13.1). Allylic oxidation forms several mid-chain conjugated dienols, including 5-, 8-, 9-, 11-, 12-, and 15-HETEs. Omega-terminal (ω/ω-1)-hydroxylation forms C16–C20 alcohols of AA (16-, 17-, 18-, 19-, and 20-HETEs). Olefin epoxidation by CYP epoxygenases results in the production of four regioisomeric *cis*-epoxyeicosatrienoic acids (EETs; 14,15-, 11,12-, 8,9-, and 5,6-EETs) (Fig. 13.2). Studies have demonstrated that these CYP-derived eicosanoids also have a multitude of potent biological activities [3].

13.1.2 Role of Phospholipase A_2 in Eicosanoid Biosynthesis

The initial step in eicosanoid production by CYPs, COX, and LOX enzymes is liberation of polyunsaturated fatty acids (PUFAs), such as AA, from plasma membranes. Fatty acids *in vivo* are primarily esterified to the *sn*-2 position of cell membrane glycerophospholipids [1]. These fatty acids act as important structural components that regulate membrane fluidity and permeability. Storage of phospholipid-bound fatty acids in the membrane also provides a reservoir for lipids during the initial step in eicosanoid biosynthesis [1, 4, 5]. Physiological stressors such as ischemia or inflammation can activate phospholipase A_2 (PLA_2) enzymes that cleave AA from the phospholipid and make it available for oxidative metabolism by the three major enzyme systems (Fig. 13.1) [6–9].

D. C. Zeldin (✉) · M. L. Edin · J. Cheng · A. Gruzdev · S. L. Hoopes
Division of Intramural Research, National Institute of Environmental Health Sciences, Building 101, Room A214, 111 T.W. Alexander Drive, Research Triangle Park, NC 27709, USA
e-mail: zeldin@niehs.nih.gov

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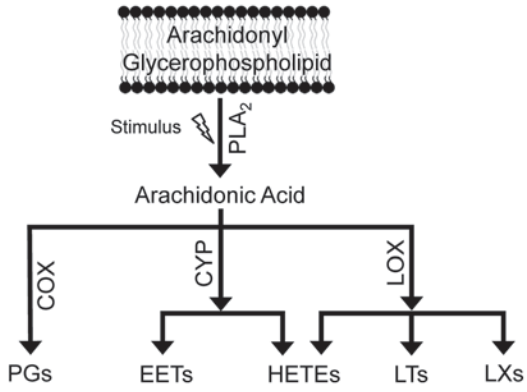

Fig. 13.1 Arachidonic acid is released from lipid bilayers by phospholipase A_2 (*PLA_2*) and then metabolized by cyclooxygenase (*COX*), cytochrome P450 (*CYP*), and lipoxygenase (*LOX*) enzymes to form prostaglandins (*PGs*), epoxyeicosatrienoic acids (*EETs*), hydroxyeicosatetraenoic acids (*HETEs*), leukotrienes (*LTs*), and lipoxins (*LXs*)

Three categories of PLA_2 enzymes that regulate AA release are classified based on their primary structure, cellular localization, and requirement for Ca^{2+}. Exposure of cytosolic PLA_2 ($cPLA_2$) to micromolar concentrations of Ca^{2+} induces its translocation to the surface membrane and enzyme activation. Secretory PLA_2 ($sPLA_2$) primarily functions extracellularly and is activated by millimolar concentrations of calcium. Ca^{2+}-independent PLA_2 ($iPLA_2$) is expressed intracellularly and may be regulated by ATP, caspase cleavage, calmodulin, or protein aggregation [6, 10].

Studies with PLA_2 inhibitors in mouse models and primary human samples reveal the critical role of this enzyme in eicosanoid-regulated vascular biology. $cPLA_{2\alpha}$-deficient mice exhibit reduced PG production and inflammatory responses [11]. $sPLA_2$ expression is low in most tissues, but it is increased in plasma of patients

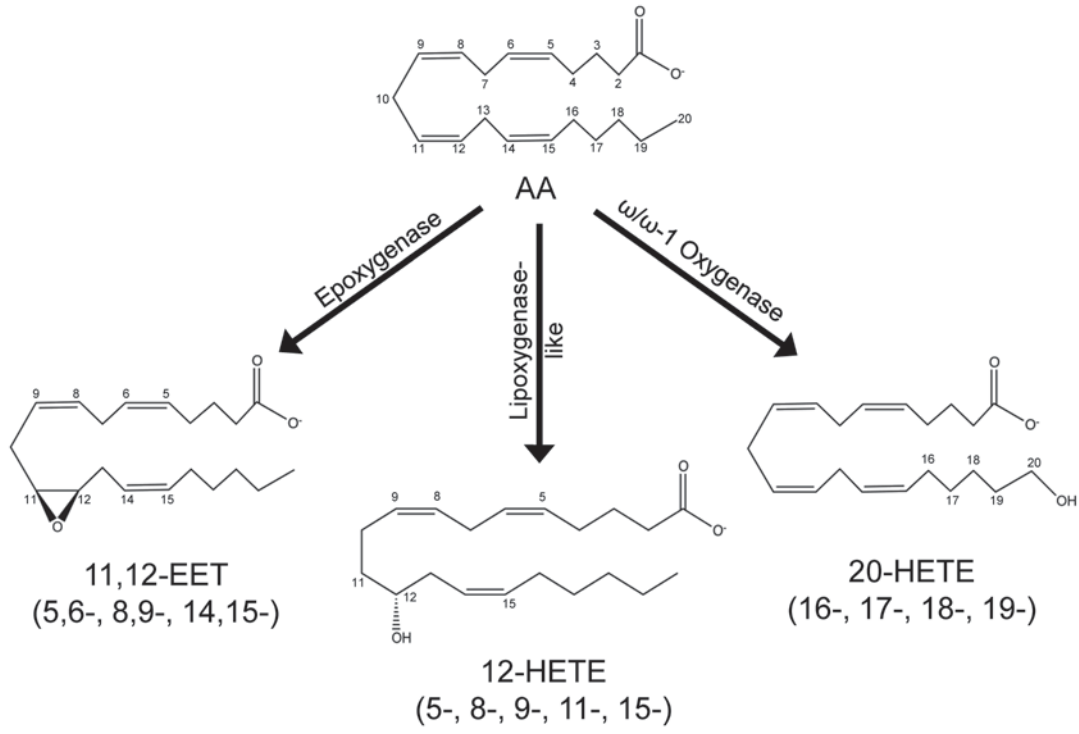

Fig. 13.2 Arachidonic acid (*AA*) is metabolized by cytochrome P450 (*CYP*) monooxygenases in epoxygenase, lipoxygenase-like, and ω/ω-1 hydroxylase reactions to form epoxyeicosatrienoic acids (*EETs*), mid-chain hydroxyeicosatetraenoic acids (*HETEs*), and ω/ω-1 HETEs, respectively

with elevated cardiovascular risk and is observed in human atherosclerotic lesions [12]. Transgenic overexpression of $sPLA_2$ increases atherosclerotic development in mice [13]. Diminished cell proliferation and motility of $iPLA_2$-deficient smooth muscle cells are associated with decreased AA release and PG production [14].

13.2 CYP Peroxide Isomerases

13.2.1 Prostacyclin and Thromboxane Synthases

Free AA is metabolized by PG-endoperoxide synthases (or COXs) to produce a variety of prostanoids, including PGs, PGI_2, and TXA_2. Both the constitutively expressed COX-1 and the inducible COX-2 enzymes convert AA to PGH_2 via two distinct but mechanistically linked stages, each catalyzed by a different activity site. The COX site reacts AA with two O_2 molecules to produce PGG_2, which has an endoperoxide ring and a hydroperoxide group. The hydroperoxide group is then reduced in the peroxidase site forming PGH_2, which is metabolized by secondary PG synthases to form numerous PGs, including PGD_2, PGE_2, PGF_{2a}, PGI_2, and TXA_2. The synthase responsible for generation of PGI_2 from PGH_2 is prostacyclin synthase (PTGIS), a member of the CYP superfamily (CYP8A1). Similarly, the TXA_2 synthase (TXAS) is also known as CYP5A1 (Fig. 13.3).

Like other mammalian CYPs, PTGIS and TXAS are membrane-bound hemoproteins; however, both enzymes lack typical CYP monooxy-

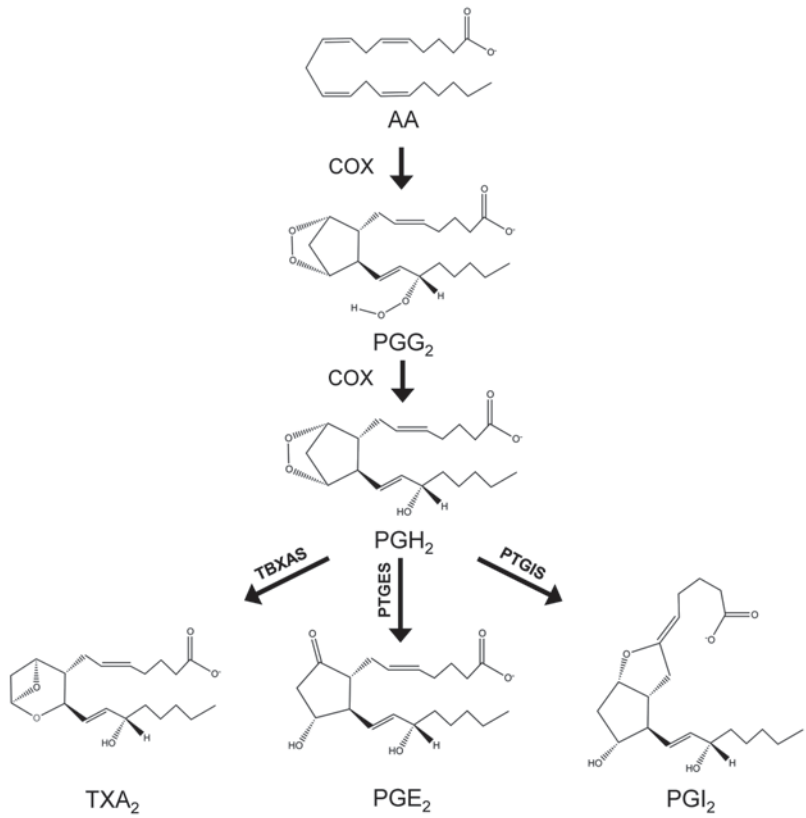

Fig. 13.3 Arachidonic acid (*AA*) is metabolized by cyclooxygenase (*COX*) to prostaglandin (*PG*) G_2 that rearranges to PGH_2. PGH_2 is metabolized to thromboxane A_2 (*TXA_2*), PGE_2, and prostacyclin (*PGI_2*) by thromboxane synthase (*TXAS*), PGE_2 synthase (*PGES*), and prostacyclin synthase (*PTGIS*), respectively

genase activity and instead cleave the epidioxy bond of PGH_2 to form PGI_2 and TXA_2, respectively [15, 16]. PTGIS is constitutively expressed in vascular cells such as endothelial cells and smooth muscle cells. As a result, PGI_2 synthesis is highest in highly vascularized tissues such as the kidney, lung, uterus, testes, and spleen [17]. The PTGIS promoter contains multiple Sp1-binding domains that drive constitutive expression and may also be involved in the upregulation of PTGIS by inflammatory cytokines such as interleukin-6 and tumor necrosis factor alpha (TNFα) [18]. While PTGIS expression can be induced by inflammatory stimuli, its activity can be inhibited by peroxynitrite-mediated tyrosine nitration. Peroxynitrite is a reaction product of superoxide and nitric oxide. Thus, under conditions of oxidative stress, PTGIS inactivation will shift vascular prostanoid production away from PGI_2 in favor of TXA_2 because TXAS is not affected by tyrosine nitration [19].

TXAS is highly expressed in platelets, but is also found in lung, kidney, spleen, stomach, and gastrointestinal tissues [20, 21]. TXAS does not appear to be subject to posttranslational modification as enzyme activity correlates well with absolute expression levels; however, TXAS may be highly sensitive to inhibition by oxidative damage [22]. While TXAS is constitutively expressed in many tissues, differential TXAS expression has been observed during *in utero* development and in tumorigenesis, which may be regulated by NF-E2 family transcription factors [23].

13.2.2 Physiological Effects of Prostacyclin and Thromboxane

Both PGI_2 and TXA_2 have very short biological half-lives [24, 25]; however, PGI_2 and TXA_2 have potent effects on vascular tissues, a topic that has been well reviewed elsewhere [18, 23]. PGI_2 transactivates a heterotrimeric G-protein-coupled receptor (GPCR), the PGI_2 receptor (PTGIR/IP receptor), to induce signaling. IP receptors are expressed in vascular cells including endothelial and smooth muscle cells and platelets, as well as in highly vascularized tissues including the lung, heart, and kidney [26]. IP receptors mainly act through $G\alpha_s$ to increase intracellular cyclic adenosine monophosphate (cAMP), though in some tissues they may activate PLA_2 C through $G\alpha_q$ or reduce cAMP levels through $G\alpha_i$. In addition, PGI_2 can activate nuclear signaling through peroxisome proliferator-activated receptors (PPARs). Similar to PGI_2, TXA_2 signals through GPCRs; however, the TXA_2 receptors (TP receptors, TPα and TPβ) are primarily linked to $G\alpha_q$ and $G\alpha_{12/13}$. $G\alpha_q$ activates signaling through PLA_2 C, inositol triphosphate (IP_3) and diacylglycerol to increase intracellular calcium levels while $G\alpha_{12/13}$ acts through Rho family GTPases to induce cytoskeletal rearrangement [23].

PGI_2 and TXA_2 act with opposing effects in vascular tissues. Vascular responses and resolution often depend on the coordinated crosstalk between these pathways. TXA_2 potently induces platelet aggregation, while PGI_2 prevents formation of platelet aggregates to reduce thrombosis [27, 28]. Interestingly, TXA_2 generated in platelets induces PGI_2 in endothelial cells [29]. Consequently, PGI_2 induces phosphorylation of the TPα receptor to downregulate TXA_2 signaling [30]. In vascular smooth muscle, PGI_2 induces vasodilation through cAMP-dependent and/or cAMP-independent signaling to large conductance Ca^{2+}-dependent potassium channels (BK_{Ca}) or adenosine triphosphate (ATP)-sensitive potassium channels (K_{ATP}) resulting in hyperpolarization and limited intracellular Ca^{2+} [18, 28]. In contrast, TXA_2 activates $G\alpha_{12/13}$ and/or $G\alpha_q$ signaling to induce Rho- and Ca^{2+}-mediated contraction of vascular smooth muscle [31, 32]. In endothelial cells, PGI_2 induces angiogenesis, enhances tight junctions and barrier function, reduces inflammation, and limits apoptosis [33, 34], while TXA_2 increases inflammatory activation of endothelial cells [35]. PGI_2 signaling is protective in atherosclerosis models by reducing smooth muscle migration, proliferation, and hypertrophy to reduce neointima formation [36]. The pro-inflammatory actions of TXA_2 in heart, lung, and kidney contribute to the progression of allergies, asthma, renal, and cardiovascular disease [23]. TXA_2 induces angiogenesis and also promotes tumor migration and metastasis [37].

13.3 CYP Monooxygenases

13.3.1 ω-Hydroxylases

13.3.1.1 ω/ω-1 Hydroxylase Metabolism

The ω-hydroxylation of fatty acids, which involves the addition of a hydroxyl group at or near the ω-terminal carbon, was first shown to be catalyzed by the liver microsomal enzyme system in the 1960s [38]. In particular, the CO pigment of CYP was recognized as a constituent of the microsomal mixed function oxidase system that contributes to the ω-hydroxylation of steroids [39]. Substrates that are susceptible to ω-hydroxylation include laurate and AA [38]. Early reports showed that CYP enzymes catalyze the ω (C-20) and ω-1 (C-19) hydroxylation of AA [40]. In 1990, Falck et al. demonstrated that CYP enzymes also hydroxylate the C-16 (ω-4), C-17 (ω-3), and C-18 (ω-2) carbons of AA [41]. Thus, in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen, CYPs mediate the hydroxylation of AA to generate a variety of ω-terminal HETEs including 16-, 17-, 18-, 19-, and 20-HETE (Fig. 13.2).

13.3.1.2 CYP ω/ω-1 Hydroxylases

Various CYP isoforms can catalyze oxidation at C16-19 of AA (Table 13.1). Oxidation of the ω-terminal carbon (C-20) to generate 20-HETE is mostly restricted to the CYP4 family, which includes the isoforms of the CYP4A, CYP4B, and CYP4F subfamilies. CYP2C40 was also demonstrated to produce primarily 16-HETE; it metabolizes AA in a highly regio- and stereospecific manner to form 16(*R*)-HETE [42]. There is evidence that CYP1A1 and CYP1A2 are also involved in the generation of 16-HETE [41]. The formation of 17-HETE has been attributed to CYP1A, as exposure of marine fish to benzo(a) pyrene, an inducer of CYP1A, results in 17-HETE production in liver microsomes. Benzo(a) pyrene also shifts hydroxylation in favor of 19-HETE, suggesting that CYP1A can catalyze the formation of 19-HETE [43].

The metabolism of AA to 18(*R*)-HETE was first characterized in the microsomes of monkey seminal vesicles [44]. Bacterial CYP102 has been shown to catalyze the formation of nearly enantiomerically pure 18(*R*)-HETE [45]. In 1993, Laethem et al. demonstrated that CYP2E1 produces both 18-HETE and 19-HETE, with 18-HETE being 100% *R* isomer and 19-HETE being 70% *S* and 30% *R* [46]. Furthermore, a CYP2J isoform cloned from sheep liver showed a preference for 18-HETE biosynthesis (86% of total), with formation of 19- and 20-HETE also being observed [47]. In addition to CYP1A [43] and CYP2E1 [46], CYP2C19 can metabolize AA to 19-HETE [48]. In hypertrophied hearts, CYP4A2 and CYP4A3 appear to play a role in 19-HETE formation [49]. In 2001, Qu et al. identified CYP2J9 as a mouse AA ω-hydroxylase that is predominantly expressed in the brain and produces 19-HETE [50]. Most recently, Chuang et al. demonstrated that CYP2U1, a novel human thymus- and brain-specific CYP enzyme, metabolizes AA to both 19-HETE and 20-HETE [51].

With the exception of CYP2U1 [51], the formation of 20-HETE is catalyzed mainly by members of the CYP4 family, including CYP4A, CYP4B, and CYP4F subfamilies. Of these, CYP4A1 and CYP4A8 exhibit only ω/ω-1-hydroxylation activities. In human kidney microsomes, CYP4F2 is the major enzyme that metabolizes AA to form 20-HETE [52]. Iso-

Table 13.1 Cytochrome P450 (CYP) isoforms and metabolites. The CYPs that catalyze the ω-hydroxylation of arachidonic acid to 16-, 17-, 18-, 19-, and 20-HETE are displayed

Metabolite	CYP isoforms
16-HETE	CYP2C40, CYP1A1, CYP1A2
17-HETE	CYP1A
18-HETE	CYPBM-3, CYP2J, CYP1A2, CYP1A5, CYP2E1
19-HETE	CYP1A, CYP2C19, CYP2E1, CYP2J9, CYP2U1, CYP4A2, CYP4A3
20-HETE	CYP2U1, CYP4A, CYP4F

forms of CYP4F are found in rat kidneys, mouse glomeruli, rabbit aortic vascular smooth muscle cells, human kidneys, and human livers [53–56]. Isoforms of CYP4A are predominantly found in humans, rats, mice, and rabbits [53, 57]. In the rat kidney, CYP4A1, CYP4A2, and CYP4A8 are highly expressed in the renal proximal tubules and vasculature [56, 58].

13.3.1.3 Physiological Effects of ω-Terminal HETEs

The effects of 16-, 17-, 18-, 19-, and 20-HETE have been studied to varying degrees. Activated polymorphonuclear leukocytes (PMNs) are known to produce 16-HETE. In vitro, 16(*R*)-HETE inhibits human PMN adhesion and aggregation. Administration of 16-HETE to rabbits with thromboembolic stroke leads to reduction in intracranial pressure [59]. Synthesis of leukotriene B_4, a pro-inflammatory molecule, is also increased by 16(*R*)-HETE. Carroll et al. demonstrated that 16(*R*)-HETE promotes vasodilation of renal arteries in a COX-dependent manner [60]. Furthermore, 16(*S*)-HETE inhibits the activity of the adenosine triphosphatase (ATPase) in the renal proximal tubule [60]. Similarly, 17(*S*)-HETE inhibits proximal tubule ATPase activity while 17(*R*)-HETE is inactive in this system.

18(*R*)-HETE, 19(*S*)-HETE, and 19(*R*)-HETE all increase vasodilation of renal arteries in rabbits [60]. Zhang et al. demonstrated that both 18(*R*)-HETE and 19(*R*)-HETE blunt the sensitizing effect of 20-HETE on phenylephrine-induced constriction of renal interlobar arteries in spontaneously hypertensive rats [61]. Escalante et al. showed that 19(*S*)-HETE is a potent stimulator of renal Na^+/K^+-ATPase activity [62]. The earliest report documenting the vasoconstrictor activity of 19-HETE was published in 1989 by Escalante et al. [63]. 19-HETE and 20-HETE increase the magnitude of K^+-induced vasorelaxation responses in rat aortic rings in a COX-dependent manner [64]. In rabbit proximal tubules, 19(*S*)-HETE promotes volume absorption [65]. In the mouse brain, 19-HETE alters neurotransmitter release by inhibiting the activity of P/Q-type Ca^{2+} channels [50]. Furthermore, both 18(*R*)-HETE and 19(*R*)-HETE can block the effects of 20-HETE in the vasculature, suggesting that it may compete for binding to the same receptor, which has yet to be identified [61, 66].

It is well known that 20-HETE has opposing effects depending on its site of action. It plays an antihypertensive role in renal tubules, where it promotes water and Na^+ excretion. In proximal tubules, 20-HETE induces phosphorylation of the Na^+/K^+-ATPase alpha subunit via protein kinase C (PKC) to inhibit Na^+/K^+-ATPase activity [67]. In the medullary thick ascending limb, it inhibits the large-conductance 70 pS K^+ channel and the Na^+-K^+-$2Cl^-$ cotransporter to prevent K^+ efflux and Na^+ reabsorption [68]. In the vascular system, excluding the pulmonary microcirculation, 20-HETE promotes hypertension by uncoupling endothelial nitric oxide synthase to decrease nitric oxide bioavailability, increasing the generation of reactive oxygen species (ROS), enhancing vasoconstriction responses, and impairing vasorelaxation responses [66, 69, 70]. 20-HETE increases vasoconstriction via PKC-dependent mechanisms [71] and these effects have been attributed to the renin-angiotensin system [72, 73]. In addition, 20-HETE induces angiogenesis and proliferation in endothelial cells, endothelial progenitor cells, and glioma cells [74, 75], and it may play a role in the development of tumors and cancer. In mouse lungs, 20-HETE mediates ozone-induced, neutrophil-independent airway hyperresponsiveness through mechanisms that are not yet clear.

13.3.2 CYP Mid-Chain Hydroxylases

13.3.2.1 Lipoxygenase-Like Reaction

CYP monooxygenases can catalyze *bis*-allylic oxidation (LOX-like reaction) to generate six regioisomeric HETEs (5-, 8-, 9-, 11-, 12-, and 15-HETE). The mechanism for CYP-dependent HETE formation involves oxidation of C7, C10, or C13, followed by acid-catalyzed rearrangement to the corresponding *cis*- or *trans*-dienols [76, 77]. The initial finding that CYP-derived 12-HETE formation was predominantly 12(*R*)-HETE suggested that CYPs generated enantiomers different from those produced by 12-LOX

enzymes, which are known to mostly produce 12(*S*)-HETE. However, 12(*R*)-HETE-producing 12-LOX enzymes were later identified [78, 79]. Various LOXs are capable of producing 5-, 8-, 12-, and 15-HETE, and aspirin-treated COXs can also produce 11(*R*)-, 15(*R*)-, and 15(*S*)-HETE [80–82]. While some effects can be traced to CYP-dependent HETE formation, it is unclear to what degree *bis*-allylic oxidation of AA by CYPs contributes to the overall production and biological actions of these HETEs [83].

13.3.2.2 Mid-Chain Hydroxylases

CYP metabolism of AA to ω-hydroxy and epoxy eicosanoids has been more intensely studied than CYP metabolism of AA to mid-chain HETEs; however, *bis*-allylic oxidation of PUFAs by CYP enzymes has been observed. CYP1A1, CYP1A2, CYP3A4, CYP2C8, CYP2C9, and CYP2C19 are modest producers of mid-chain HETEs [48, 84]. While CYP2C8 and CYP2C9 predominantly generate epoxides from AA, they can also produce a significant amount of 15- and 12-HETE, respectively. The production of 12-HETE by CYP2C9 is almost entirely (>95%) 12(*R*)-HETE [48]. Human CYP1B1 predominately produces mid-chain HETEs (54% of total AA products), including 5-, 8-, 12-, and 15-HETE [84]. While CYP2J2 predominantly produces epoxides (76% of all metabolites), it also produces both 8-, and 15-HETE. Interestingly, regioselective generation of mid-chain HETEs is preserved in many murine CYP isoforms. Murine CYP1B1 also produces high percentage of 5-, 8-, and 12-HETE. Multiple murine CYP2C isoforms produce 12- and 15-HETE, and murine CYP2J isoforms are most likely to produce the 8- and 15-HETEs.

13.3.2.3 Mid-Chain HETE Effects

The (*R*)-HETEs are known to have potent biological effects; however, it is typically unknown whether COX, LOX, or CYP enzymes are responsible for (*R*)-HETE generation. 12(*R*)-HETE formed in corneal epithelium is believed to be of CYP origin and inhibits the Na^+/K^+-ATPase to regulate ocular transparency and aqueous humor secretion [86]. Inhibition of the Na^+/K^+-ATPase by 12(*R*)-HETE also increases urinary sodium and potassium excretion, and urine volume. 5(*R*)- and 12(*R*)-HETE are more potent than their corresponding (*S*) enantiomers in promoting neutrophil migration [85, 86]. 5-, 12-, and 15-HETE induce cell proliferation in a variety of cell types, while 8-, and 11-HETE display antiproliferative effects [87]. Additional studies are required to clarify whether these HETEs are generated by CYP or other enzyme systems.

13.3.3 CYP Epoxygenases

13.3.3.1 CYP-Dependent Biosynthesis of EETs

In the early 1980s, the first evidence for CYP-dependent generation of EETs from AA was detected in kidney and liver microsomes [40, 88]. CYP enzymes are capable of incorporating oxygen into each olefin of AA to generate all four regioisomeric *cis*-EETs (5,6-, 8,9-, 11,12-, and 14,15-EET) [88]; however, many tissue microsomes or recombinant CYP enzymes show a preference for generation of 14,15- and 11,12-EET over other regioisomers [88, 89]. NADPH-dependent CYP metabolism of AA generates exclusively *cis*-EETs [90, 91], whereas hydroperoxide-dependent CYP oxidation of AA can result in formation of both *cis*- and *trans*-EETs [92, 93]. *Trans*-EETs are found *in vivo*, and possess signaling capabilities [93, 94].

CYPs can generate all four EET regioisomers as either (*S, R*) or (*R, S*) stereoisomers. The ratio of (*R, S*) to (*S, R*) isomers varies between CYPs and between different regioisomers produced by the same CYP. For instance, CYP2C8 selectively produces (*R, S*) enantiomers of both 14,15-EET and 11,12-EET [90, 95]. In contrast, CYP2J2 produces 14(*R*),15(*S*)-EET over 14(*S*),15(*R*)-EET, but generates roughly equal amounts of each 11,12-EET stereoisomer [96]. CYP2C9 displays a modest preference for 14(*R*),15(*S*)-EET, but generates more 11(*S*),12(*R*)-EET than 11(*R*),12(*S*)-EET [95]. Information on the biological effects of EET stereoisomers is limited, as many studies have used racemic EET mixtures. However, (*R,S*) and (*S, R*) stereoisomers are known to have different effects in some systems. For ex-

ample, only the 14(*R*),15(*S*)-EET stereoisomer inhibits COX activity [97], while 14(*S*),15(*R*)-EET is more potent in dilation of bovine coronary arteries [98]. Only 11(*R*),12(*S*)-EET dilates small renal arterioles at low concentrations [98], whereas only the 8(*S*),9(*R*)-EET enantiomer is a renal vasoconstrictor. The 14(*R*),15(*S*)-EET stereoisomer binds the membrane-binding site on U937 cells more readily than 14(*S*),15(*R*)-EET, and thus may be the more potent agonist for the putative EET receptor [99]. Importantly, some discrepancies in physiological responses to EETs have been observed. For example, EETs are generally thought to be vasodilatory in the context of blood vessels; however, differences between species and in different vascular beds have yielded varying results [100–106].

13.3.3.2 CYP Epoxygenases

Generation of EETs has been demonstrated in numerous tissues, including liver, kidney, lung, skin, heart, brain, adrenal, pituitary, ovaries, and blood vessels [40, 88, 96, 107–114]. EETs are produced by numerous cell types, including endothelial cells, cardiomyocytes, astrocytes, and cancer cells [115–118]. The term "epoxygenase" is used to describe CYPs that generate epoxides from PUFAs. The majority of CYP epoxygenases belong to the CYP2 family, in particular the CYP2C and CYP2J subfamilies; however, numerous CYPs can generate detectable amounts of EETs.

Many human CYPs and their orthologs in other species are known to generate EETs. Purified CYP1A1, CYP1A2, CYP2B6, and CYP2E1 primarily generate HETEs from AA but also produce EETs [84, 119]. In humans, CYP2C8, CYP2C9, and CYP2J2 appear to be the most important AA epoxygenases. CYP2C8 metabolizes AA exclusively to 14,15- and 11,12-EET at high rates [48, 119]. CYP2C8 is abundantly expressed in heart, liver, kidney, and intestines, but is also found in blood vessels to varying degrees [120, 121]. CYP2C9 generates 14,15-, 11,12-, and 8,9-EETs at slightly higher rates than CYP2C8 [95, 119]. CYP2C9 is thought to be the predominant AA epoxygenase in human aorta and coronary arteries [120]. It also is highly expressed throughout the gastrointestinal system, liver, heart, pancreas, kidney, adrenal, pituitary, lymph nodes, lung, and skin [121]. CYP2C19 generates 14,15- and 8,9-EET, but is not considered a major human epoxygenase [48]. CYP2J2 also metabolizes AA producing primarily epoxygenase products, including all four EET regioisomers, but favoring the production of 14,15-EET [96]. CYP2J2 expression is highest in the heart, gastrointestinal system, liver, pancreas, kidney, and adrenal tissues, but is also expressed in blood vessels [96, 120, 121]. Rodent homologs of CYP4X1 are highly expressed in the brain, but are also present in the lung, liver, and kidney [122, 123]. CYP4X1 efficiently metabolizes AA to EETs (Edin and Zeldin, unpublished observations) and anandamide to 14,15-EET ethanolamide [124].

Identification of rodent homologs to human CYP epoxygenases is complicated by gene duplication events. For instance, while the human genome contains four CYP2C and one CYP2J subfamily members, mice have fifteen CYP2C and eight CYP2J subfamily members. Of these, at least eight CYP2C (2C29, 2C38, 2C39, 2C40, 2C44, 2C50, 2C54, and 2C55) and all eight CYP2J (2J5, 2J6, 2J8, 2J9, 2J11, 2J12, and 2J13) isoforms produce EETs [125–128]. Rat and mouse both express a homolog to human CYP4X1 [122, 123, 129]. Rat CYP2 family members known to produce EETs include CYP2B1, CYP2B2, CYP2C2, CYP2C10, CYP2C11, CYP2C23, CYP2C24, CYP2J3, and CYP2J4 [130–132]. Known rabbit epoxygenases include CYP2B4, CYP2B5, CYP2C1, CYP2C4, and possibly CYP2J1 [131, 132]. In most instances, defining the homologs to human CYP2C members or to CYP2J2 is problematic due to differences in expression patterns and/or metabolic profiles [133].

13.3.3.3 Biological Fate of CYP-Derived EETs

After oxygenation, the fate of fatty acid epoxides is diverse and varied (Fig. 13.4). EETs may transactivate membrane receptors or directly bind to ion channels or other proteins to cause biological effects. All four EET regioisomers can be esterified into phospholipids in cell mem-

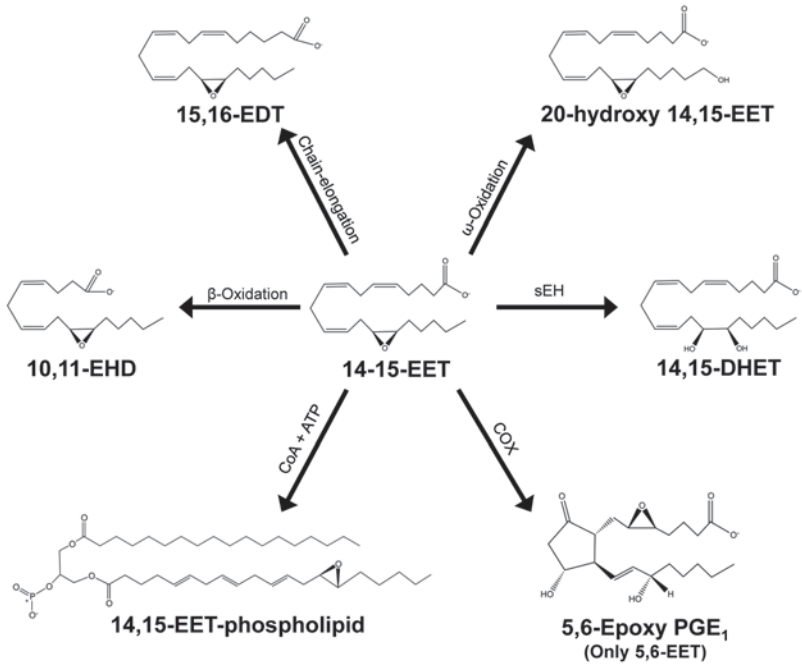

Fig. 13.4 Fatty acid epoxides, such as 14,15-epoxyeicosatrienoic acid (*14,15-EET*), are metabolized by multiple pathways. EETs can undergo additional oxygenation by CYPs or cyclooxygenases (*COXs*), can be hydrolyzed to dihydroxyeicosatrienoic acids (*DHETs*) by epoxide hydrolases such as soluble epoxide hydrolase (*sEH*), or reesterified to the plasma membrane in an Acyl-CoA dependent process. EETs can also undergo chain shortening or elongation to epoxyhexadecadienoic acids (*EHD*) or epoxydocosatrienoic acids (*EDTs*), respectively

branes of most tissues, including the heart [96], liver [134], and kidney [135]. EETs become esterified through a coenzyme A (CoA)-dependent process [103, 136, 137]. EETs are incorporated into phospholipids primarily at the *sn*-2 position [137, 138]. Membrane incorporation of AA is preferred over EETs. Among the EETs, incorporation into membranes is highest for 5,6-EET, intermediate for 8,9- and 11,12-EET, and lowest for 14,15-EET [4, 139]. Esterification into membrane phospholipids suggests that membranes may contain a store of EETs available for later release [103, 137, 138].

An important pathway for metabolism of EETs is hydrolysis to dihydroxyeicosatrienoic acids (DHETs) by epoxide hydrolases (EHs or EPHXs). There are at least five mammalian enzymes thought to contain EH activity: EPHX1, EPHX2, EPHX3, EPHX4, and PEG1/MEST [140]. Of these, soluble epoxide hydrolase (sEH/EPHX2) is the most active for EET hydrolysis [141]. Compared to EETs, DHETs often show diminished activity in biological assays [105, 142, 143]; however, there are several notable exceptions, including maintenance of vasodilatory properties and agonism of the peroxisome proliferator-activated receptor (PPAR) [102, 105, 144, 145]. Hydrolysis also speeds elimination of EETs since DHETs are released from cells and are not reincorporated into phospholipid in membranes. Both EETs and DHETs are found in blood, but only DHETs are detectable in urine, suggesting a process of selective elimination [146, 147]. Studies with selective sEH inhibitors or genetic disruption of sEH in mice leads to a significant increase in plasma levels of EETs, a reduction in plasma levels of DHETs, and many physiological changes associated with increased EETs [148, 149]. Given the beneficial preclinical data of CYP-derived EETs in cardiovascular diseases, pharmacological inhibition of sEH has promising therapeutic potential.

Hydrolysis of EETs to DHETs appears to be a primary mechanism of EET removal; however, in cells with low EH expression or during pharmacological sEH inhibition, EET chain shortening or elongation can be observed [150, 151]. Acetyl-CoA ligation is the initial step in the process, after which EETs may be elongated to 22-carbon epoxides and can be reincorporated into plasma membranes [152]. EETs may also be shortened through β-oxidation to 16-carbon epoxides that may maintain physiological functions or undergo further truncation [150, 153]. Conjugation of EETs to glutathione can be detected in various cellular systems; however, it is unclear if significant amounts of glutathione conjugation occur at physiological EET levels [154]. EETs may also be bound by fatty acid-binding proteins (FABPs). FABPs display a higher affinity for EETs than DHETs and may limit EET hydrolysis by sEH and/or EET release and signaling [155, 156].

EETs can also be further metabolized by CYPs or other enzymes. For example, 8,9-, 11,12-, and 14,15-EET can undergo ω-hydroxylation by CYP4A enzymes [157]. In addition, 5,6- and 8,9-EET can serve as substrates for COXs to yield epoxy PGs or other metabolites with vasoactive and mitogenic properties [158, 159].

13.3.3.4 Biological Actions of CYP-Derived EETs

13.3.3.4.1 Cellular Targets

The identity of a membrane-bound EET receptor remains elusive despite strong evidence that EETs transactivate a GPCR. Radioligand-binding assays with 14,15-EET show a selective membrane-binding site on EET-responsive monocytic cells [160, 161]. EETs induce cAMP accumulation in these cells, which suggests activation of a canonical GPCR pathway [99]. EETs covalently bound to silica beads are able to transactivate aromatase transcription without entering vascular smooth muscle cells [162]. EETs appear to directly bind and activate Kir6.1-containing ATP sensitive potassium (K_{ATP}) channels, while activation of Kir6.2-containing K_{ATP} channels by EETs requires activation of protein kinase activity (PKA) [163]. Several groups have attempted to identify the putative EET receptor with no definitive success [161, 164]. EETs have been shown to act as modest antagonists to the TXA_2 receptor [165]. Others report strong, selective activation of the PGE_2 receptor subtype EP2 [166]. However, additional and conflicting studies have failed to confirm whether either receptor is responsible for EET-dependent signaling [161, 165, 166].

13.3.3.4.2 Vascular Tone

Treatment of coronary arteries with AA induces potent vasodilation. This vasodilation is dependent on AA metabolism in the endothelium as endothelial-denuded vessels do not relax in response to AA [167, 168]. Inhibitor studies revealed approximately half of this vasorelaxation was induced through COX-dependent metabolism to PGI_2 and half was induced by CYP-dependent metabolism to EETs [109]. In intact vessels, agonists such as acetylcholine or bradykinin induce EET formation in endothelial cells that act as paracrine messengers to hyperpolarize underlying smooth muscle cells to induce vasorelaxation [167, 168]. Thus, EETs are identified as an endothelial-derived hyperpolarization factor (EDHF). Selective inhibitors reveal the vasodilatory roles of CYP2 subfamily enzymes, including CYP2B6, CYP2C8, CYP2C9, and CYP2J2 in humans, CYP2C34 in pigs, and CYP2C11, CYP2C23, and CYP2J4 in rats [169].

The effect of EETs as EDHFs is inhibited by iberiotoxin, which inhibits activation of large-conductance Ca^{2+}-activated potassium channels (BK_{Ca}) [105, 170]. Opening of BK_{Ca} channels allows influx of potassium and hyperpolarization of the plasma membrane that ultimately limits calcium influx and the actin/myosin cross-bridging required for smooth muscle contraction [171]. The initial mechanism through which EETs induce hyperpolarization of smooth muscle cells is less clear as EETs do not directly activate BK_{Ca} channels [100, 172]. EETs may transactivate a yet-to-be-identified GPCR [172, 173] in order to increase BK_{Ca} channel opening probability [174]. Alternatively, EETs may activate vanilloid transient receptor potential 4 (TRPV4) channels leading to small calcium transients that

activate BK_{Ca} channels to hyperpolarize cells and diminish intracellular calcium levels [175].

Both 8,9- and 11,12-EET regioisomers induce significant vasodilation at concentrations as low as 100 nM, while 14,15-EET induces vasodilation at higher concentrations (1 mu) [98, 105]. DHETs are generally less vasoactive. In human coronary arteries, 8,9- and 14,15-DHET are approximately 100-fold less potent vasodilators than their corresponding EET regioisomers [105]. In contrast, 11,12-DHET displays equal vasodilatory potential as 11,12-EET. 5,6-EET also induces vasodilation; however, its vasodilatory actions are sensitive to COX inhibition, which suggests that relaxation is dependent on additional metabolism by COXs or via stimulation of PGI_2 or PGE_2 release [176–178]. While not widely studied, the vasoactive effects of EETs are stereoselective. For example, only 11(*R*),12(*S*)-EET is vasodilatory in renal arterioles [102].

CYP expression in endothelial cells does not always elicit vasodilatory responses. In the pulmonary vasculature, EETs are potent vasoconstrictors [179]. CYP2C enzymes also produce physiologically relevant levels of ROS during fatty acid oxidation [180]. CYP2C-derived ROS limits EDHF- or nitric oxide-mediated vasodilation [181–183].

13.3.3.4.3 Inflammation

EETs are also known to have potent anti-inflammatory effects. Pretreatment of endothelial cells with EETs blocks upregulation of pro-inflammatory adhesion molecules by cytokines, including TNFα or IL-1α [142]. Overexpression of CYP2J2 or CYP2C8 in endothelial cells or global disruption of sEH increases EETs and attenuates the vascular inflammatory response to lipopolysaccharide, leading to reduced adhesion molecule expression, cytokine production, and infiltration of cells into the lung [184]. These effects appear to be largely due to inhibition of NF-κB activation, which may be subsequent to EET activation of PPARγ [185]. Interestingly, while EETs have vasodilatory/antihypertensive effects, their anti-inflammatory properties appear to prevent mortality from lipopolysaccharide-induced hypotension in a mouse model of systemic shock [186].

13.3.3.4.4 Cell Proliferation, Migration, and Apoptosis

Hypoxia induces CYP2C enzymes and increases EET production in endothelial cells [187]. EETs have potent proliferative, migratory, and angiogenic effects [188, 189]. EETs induce responses through many signaling pathways. EETs transactivate growth factor receptors such as epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF) to activate effector pathways including PI3K/AKT, MAPK, Rac, or Src, resulting in endothelial proliferation, migration, and angiogenesis [187, 190–194]. Thus, CYP epoxygenases increase angiogenesis-dependent physiological responses, including wound healing, organ regeneration, primary tumor growth, and metastasis [190, 191, 195]. Human tumors express higher levels of CYP2J2 than adjacent normal tissues and expression of CYP2J2 in cancer cells results in increased tumor growth and metastases [118, 196]. As in endothelial cells, EETs induce proliferation and migration in tumor cells [118, 190, 191, 197]. In both endothelial and cancer cells, EETs prevent apoptosis in response to intrinsic or extrinsic stimuli [118, 198, 199]. In contrast to their effects on endothelial cells, EETs reduce smooth muscle cell migration though activation of cAMP and PKA [200].

13.3.3.4.5 Ischemic Protection

EETs are protective against ischemic events in both the heart and brain. In the heart, overexpression of CYP2J2, genetic disruption of sEH, or exogenous EET treatment improves recovery of function and prevents tissue death after cardiac ischemia [117, 148, 201, 202]. Protection against cardiac injury by EETs involves preservation of cardiomyocyte mitochondria after ischemia-reperfusion. Reactive oxygen species generated during postischemic reperfusion leads to lipid, protein, and DNA peroxidation. Mitochondria serve critical roles in cell survival, death, ATP production, and apoptosis. EETs activate several pathways, including PKA, MAPK, PI3K/AKT,

and PKC, which can result in phosphorylation of an inhibitory site on glycogen-synthase-kinase 3β (GSK3β) [117, 148, 203, 204]. Inhibition of GSK3β limits opening of the mitochondrial permeability transition pore (mPTP) and prevents loss of mitochondrial membrane potential, leakage of calcium and solutes, and collapse of the electron transport chain [204, 205]. The cardioprotective effect of EETs in the heart has been shown to be reversed by PI3K and MAPK inhibitors [117, 148, 204]. EETs may also activate either sarcolemmal ($sarcK_{ATP}$) or mitochondrial K_{ATP} ($mitoK_{ATP}$) channels to protect hearts against ischemia. Openers of $sarcK_{ATP}$ channels shorten cardiac action potential duration and reduce calcium overload during ischemia [206]. $mitoK_{ATP}$ channels are also implicated in EET-induced improvement in recovery of heart function after ischemia [148]. $mitoK_{ATP}$ opening may prepare mitochondria for ischemia by inducing partial depolarization of the mitochondrial membrane, inducing transient swelling, reducing calcium overload, or altering production of ROS; however, the exact mechanisms whereby EETs elicit these effects remain unknown [206, 207]. In humans, EETs are likely generated by CYP2J2, which is highly expressed in cardiomyocytes [96, 120].

CYP epoxygenase products also mitigate damage from cerebral ischemia. Increased EETs lead to increase cerebral flow during cerebral infarction, either through neurogenic or endothelial-derived vasodilation in the brain [208–210]. EETs exhibit a wide array of potentially beneficial actions during a stroke, including vasodilation, neuroprotection, enhanced angiogenesis, and suppression of oxidative stress and postischemic inflammation [189, 211–213].

While EETs regulate ischemic damage in animal models of ischemia-reperfusion, they may also help resolve thrombotic blockage of arteries *in vivo*. EETs prevent platelet aggregation independent of effects on TXA_2 biosynthesis [97, 212]. EETs induce membrane hyperpolarization and reduce Ca^{2+} entry into platelets to inhibit platelet activation, cytoskeletal rearrangement, aggregation, and adhesion to endothelial cells [214, 215].

13.3.3.4.6 Renal Function

Multiple CYP epoxygenases are expressed in the renal vasculature and kidney tubules. Human CYP2C8, CYP2C9, and CYP2J2 enzymes are expressed in both distal and proximal renal tubules and collecting ducts [121]. Rat CYP2C11, CYP2C23, and CYP2C24 as well as murine CYP2C29, CYP2C38, CYP2C39, CYP2C44, CYP2J5, CYP2J8, CYP2J11, and CYP2J13 are also detected in kidney [125, 216]. Regulation of CYP2C, CYP2J, and sEH enzymes in rodent models of hypertension suggest that these pathways play an integral role in renal homeostasis. CYP2C inhibition causes dietary salt-sensitive hypertension; therefore, increased CYP2C expression and activity after high salt treatment is likely a compensatory response [217].

CYP-derived EETs alter kidney function through regulation of vascular tone, salt handling, and inflammation. EDHF effects of EETs dilate renal arteries and afferent arterioles to increase glomerular flow [102]. EETs inhibit the epithelial Na^+ channel (ENaC) to increase salt excretion [216]. Both effects are antihypertensive. Anti-inflammatory and antiproliferative effects of epoxides may also protect against development of end-stage renal diseases. Angiotensin II exerts hypertensive effects partly through upregulation of sEH. In addition, sEH inhibitors protect against end-organ kidney damage in a variety of pre-clinical models [218]. Thus, manipulation of the CYP epoxygenase pathway may offer promise as a treatment of renal diseases in humans.

13.3.4 Regulation of CYPs

The activity of CYP enzymes can be regulated by several factors that consequently affect the production of HETEs and EETs. Ethanol is a prominent inducer of CYP2E1, which forms 18- and 19-HETE [219]. In the rabbit kidney, deoxycorticosterone acetate induces ω/ω-1 oxygenase activity in a time-dependent and selective manner [220]. Physiological conditions including fasting and diabetes also regulate CYP2E1 in rodents, and high fat diets and palmitic acid induce CYP2E1 in human hepatocytes [221].

Clofibrate induces CYP4A activity and 20-HETE biosynthesis in Dahl salt-sensitive rats [222], whereas high-fat diet decreases CYP4A levels in rats [223]. Administration of high-salt diet to normotensive rats increases CYP4A2, CYP4A3, and CYP4A8 expression in mesenteric vessels, and administration of low-salt diet increases CYP4A3 expression [224]. In humans, CYP2E1 and CYP4F2 expression are associated with the accumulation of cadmium and lead that correlate positively with age [225]. Hormones are also involved in the regulation of CYPs and the release of HETEs. Angiotensin II induces the release of 16-, 17-, 18-, 19-, and 20-HETE from the isolated perfused rabbit kidney [60, 226]. Treatment of rats with 5α-dihydrotestosterone increases CYP4A activity, leading to enhanced 20-HETE production [69].

Benzo(a)pyrene and TCDD are inducers of CYP1A1, CYP1B1, and CYP4A1 enzymes [227]. CYP2C40, an inducer of 16-HETE, appears to be regulated in cystic fibrosis (CF) because mice deficient in the CF transmembrane conductance regulator had a 50% decrease in CYP2C40 levels, suggesting that the pathophysiology of CF modulates CYP2C expression [228]. In murine models of diabetes, CYP2C40 expression is decreased following induction of the diabetic phenotype [229]. Furthermore, isoniazid, an organic compound used in the treatment of tuberculosis, upregulates CYP2E1 and downregulates CYP4A expression in rat liver [230]. Interestingly, CYP2E1 expression in the kidney is unaffected by isoniazid, thus indicating that the regulation of CYP enzymes is tissue specific.

Several common single nucleotide polymorphisms (SNPs) regulate human CYP expression and metabolism. A polymorphism that substitutes valine-433 with methionine in CYP4F2 reduces 20-HETE production and increases risk for hypertension and stroke [231]. The *CYP2J2*7* promoter SNP (G-50T) disrupts an *Sp1*-binding site and reduces CYP2J2 expression [232]. The *CYP2C8*3* SNP (lysine-339 to arginine) reduces CYP epoxygenase activity [233]. Both the *CYP2J2* and *CYP2C8* polymorphisms are associated with increased risk of cardiovascular disease [234].

Acute inflammation significantly alters CYP expression and activity. Inflammatory cytokines such as TNFα suppress hepatic CYP expression at the mRNA level [235]. CYP2J4 protein expression and epoxygenase activity are reduced in a rat model of *Pseudomonas* pneumonia [236]. Lipopolysaccharide acutely suppresses murine CYP2C44 and CYP2J5 expression and activity, while resolution of inflammation correlates with the restoration of epoxygenase expression [237].

Both endogenous and exogenous agents induce CYPs through nuclear receptors. Phenobarbital induces CYP2C expression through constitutive androstane receptor (CAR)- and pregnane X receptor (PXR)-dependent transcription [238]. Endogenous lipids (including HETEs, EETs, and DHETs) or pharmacologic agents (such as clofibrate) activate PPARα-mediated transcription of *CYP1A, CYP2A, CYP2C,* and *CYP2E* subfamily members. *CYP2C8* and *CYP2C9* are potently induced during hypoxia, possibly through hypoxia-inducible factor (HIF)-1α-dependent transcription, which enhances endothelial migration and proliferation [187].

CYP expression is also regulated by sex hormones and throughout development. In mice, CYP2J5 is increased in male compared to female kidneys [239]. Pulsatile secretion of growth hormone (GH) induces Cyp2c11 expression in male rat livers at puberty. In contrast, continuous secretion of GH in female rats leads to induction of hepatic Cyp2c12 [240]. CYPs may be alternatively regulated during the dedifferentiation associated with tumor progression. For example, CYP2J2 is often upregulated in human cancers compared to adjacent normal tissues [196, 241], whereas other CYPs are downregulated in cancer [242].

13.3.5 CYP Metabolism of Other PUFAs

In addition to AA, a 20-carbon fatty acid with four olefins in an omega-6 configuration (20:4, n-6), CYPs can utilize other PUFAs as substrates. Notably, CYPs can metabolize adrenic acid (16:2, n-6), linoleic acid (LA, 18:2, n-6), gamma

linoleic acid (18:3, n-3), epoxyeicosapentaenoic acid (EPA; 20:5, n-3), and docosahexaenoic acid (DHA; 22:6 n-3) to epoxy (Fig. 13.5) and hydroxy derivatives.

CYP epoxygenases can metabolize LA to either 9,10- or 12,13-epoxyoctadecamonoenoic acids (EpOMEs). Cellular toxicity of 9,10- and 12,13-EpOME earned them the names leukotoxin and isoleukotoxin, respectively; however, subsequent studies determined that the toxicity of these leukotoxins required hydrolysis to the corresponding 9,10- and 12,13-dihydroxyoctadecamonoenoic acids (DiHOMEs). At high levels (>10 μM), EpOME or DiHOME treatment has a variety of effects, including cytotoxicity to renal tubules [243], stimulation of ROS production [244], increased contractility in rat hearts [245], cardiodepression in dogs [246], inhibition of papillary muscle contraction, and vasoconstriction of isolated arteries [247]. Treatment with lower concentrations (250 nM) of 9,10-DiHOME induces vasoconstriction and reduces recovery of contractile function in hearts after ischemia-reperfusion. In contrast, some studies suggest that large doses of LA, EpOMEs, or DiHOMEs modestly improve basal heart contractility and leukotoxins protect renal mitochondria and sodium transport during hypoxia [248, 249].

Many CYPs metabolize EPA and DHA at rates that are similar to or higher than those for AA [250]. CYP2C8 and CYP2J2 show increased selectivity for epoxygenation of the ω-3 olefin to produce primarily 17,18-epoxyeicosatetraenoic acid (EpETE) and 19,20-epoxyeicosapentaenoic acid (EpDPE) [251–254]. CYP4A and CYP4F ω-hydroxylases also efficiently metabolize EPA and DHA to 19- and 20-hydroxyeicosapentaenoic acids (HEPEs), and 21- and 22-hydroxydocosahexaenoic acids (HDoHEs), respectively. Both EPA and DHA are ω-hydroxylated by CYP4F3B. EPA and DHA can compete with AA to be metabolized by CYP4F2 and CYP4F3B. EPA and DHA are the most potent inhibitors of 20-HETE generation from AA [255]. Interestingly, several CYPs, including CYP1A1, CYP1E1, CYP4A1, and CYP4A14 have hydroxylase activity with AA as substrate, but epoxygenase activity with EPA and DHA as substrates [253, 256–258].

Relative to the effects of CYP AA metabolites, the physiological effects of CYP ω-3 metabolites are less well studied. Vascular studies using 20-HEPE and 22-HDoHE have not been performed to date so it remains unclear whether these molecules will share the vasoconstrictive and mitogenic activities of 20-HETE. Importantly, 17,18-EEQ and 19,20-EDP appear to be far more potent

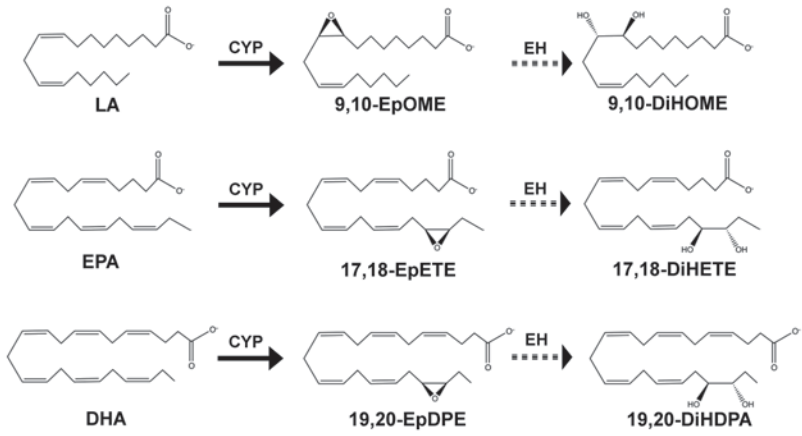

Fig. 13.5 Linoleic acid (*LA*), eicosapentaenoic acid (*EPA*), and docosahexaenoic acid (*DHA*) are all metabolized by CYP epoxygenases to form epoxyoctadecamonoenoic acids (*EpOMEs*), epoxyeicosatetraenoic acids (*EpETEs*), and epoxydocosapentaenoic acids (*EpDPEs*). Epoxide hydrolases (*EHs*) hydrolyze these epoxides to the corresponding vicinal diols, dihydroxyoctadecamonoenoic acids (*DiHOMEs*), dihydroxyeicosatetraenoic acids (*DiHETEs*), and dihydroxydocosapentaenoic acids (*DiHDPA*), respectively

vasodilators than 11,12-EET [256, 259]. The reduced vasodilation and elevated blood pressure in CYP1A1 knockout mice appears linked to the loss of ω-3 epoxide production [260]. The ability of EPA and DHA epoxides to activate K_{ATP} channels exceeds that of EETs, suggesting a role for EpETEs and EpDPEs in cardioprotection [261]. Similar to EETs, 17,18-EpETE and 19,20-EpDPE activate BK_{Ca} channels in vascular smooth muscle [256, 259]. Interestingly, different mechanisms or receptors appear to be involved because 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE), which antagonizes EET-induced vasodilation, does not alter 17,18-EpETE-induced vasodilation [262]. 17,18-EpETE also has EET-like anti-inflammatory and bronchodilatory effects in the lung [263, 264].

COXs, PTGIS, and TXAS can metabolize EPA to the PGI_2- and TXA_2-like compounds, PGI_3 and TXA_3, respectively. PGI_3 maintains potent activity compared to PGI_2, though TXA_3 is less potent than TXA_2. Thus, omega-3-rich diets may alter the ratio of prostacyclin to thromboxane signaling and help protect against vascular inflammation, hypertension, and thrombosis [18, 34].

13.4 Conclusions

The CYPs are capable of metabolizing a wide array of substrates to bioactive molecules that affect critical cellular and organ functions. Members of the CYP2, CYP4, CYP5, and CYP8 families have been shown to be involved in the activation of endogenous fatty acids. Many studies confirm the physiological importance of these pathways in cardiovascular, renal, and pulmonary homeostasis. Future studies will better define the role of CYP fatty acid metabolism in the pathophysiology of disease. Selective genetic or pharmacological manipulation of these CYP isomerase, hydroxylase, and epoxygenase pathways may represent promising avenues for treatment of human diseases.

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