Structural Diversity of Eukaryotic Membrane Cytochrome P450s*

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X-ray crystal structures are available for 29 eukaryotic microsomal, chloroplast, or mitochondrial cytochrome P450s, including two non-monoxygenase P450s. These structures provide a basis for understanding structure-function relations that underlie their distinct catalytic activities. Moreover, structural plasticity has been characterized for individual P450s that aids in understanding substrate binding in P450s that mediate drug clearance.

Structural characterization of eukaryotic membrane cytochrome P450s has focused largely on human cytochrome P450s because of their importance in human health. Human P450s are either specialists that exhibit highly conserved functions in vertebrate species or generalists that facilitate metabolic clearance of structurally diverse compounds to reduce toxic exposures, although in some cases, mutagenic or more toxic metabolites are produced. Genes encoding generalist P450s vary between closely related species, leading to functionally distinct enzymes. Orthologs typically exhibit 70% or greater identity, respectively. Orthologs typically exhibit 70% or greater sequence identity. There are 57 genes encoding human P450s comprising 18 families, including 35 genes for predominantly generalist P450s in families 1–4.

Common Features of Membrane P450 Structures

The catalytic domain of ~460 amino acids folds into a triangular prism shape (Fig. 1A) that is similar to that of soluble prokaryotic P450s (3). Twelve α-helices first identified for the structure of soluble prokaryotic 101A1 (4) are designated by letters A–L. Additionally, there is a highly conserved β-sheet domain near the N terminus of the protein. The number of helices is typically larger, but these helices are less conserved (Fig. 1). Spatial conservation is highest for the structural core of the protein and diverges most for the substrate-binding site (5, 6).

The heme prosthetic group is the catalytic center of the enzyme, where a reactive hypervalent oxo-iron protoporphyrin IX radical cation intermediate is formed for subsequent insertion of the iron-bound oxygen atom into a substrate bond (7). Substrates bind in a cavity or cleft above the surface of the heme in proximity to the reactive intermediate (Fig. 1). The thiolate side chain of a conserved cysteine binds to the axial coordination site of the iron opposite to the bound oxygen, giving rise to the unique spectral and functional properties of P450 enzymes.

Most P450s are monoxygenases, and electrons for reduction of the heme and subsequently the oxygen substrate are provided by protein partners that bind to the face of the protein proximal to the heme (8–11). Reduced adrenodoxin (12), a soluble Fe-S protein, serves as the reductant for vertebrate mitochondrial P450s, and in turn, it is reduced by the flavoprotein NADPH-adrenodoxin reductase. A structure of mitochondrial 11A1 crystallized with a tethered adrenodoxin bound to its proximal surface reveals the binding interaction between the proteins (10). Microsomal NADPH-cytochrome P450 reductase, which has an FMN and an FAD domain, provides two electrons for reduction of oxygen by microsomal P450s. The microsomal reductase has been crystallized in a closed form in which the flavodoxin-like FMN domain is positioned for reduction by the FAD domain (13) and in a more open form in which the FMN domain is more accessible for interaction with the proximal face of the P450 (8, 14, 15). Microsomal cytochrome b_{5} can also serve as a donor of the second electron, and interactions between cytochrome b_{5} and P450s can modulate rates and product profiles (16).

Helices C, D, and I–L, together with β-sheets 1 and 2, comprise the structural core that forms portions of the heme-binding site and the proximal surface where protein partners bind. Helix F, G, helix B–C, and the N- and C-terminal regions, which form the outer boundaries of the substrate-binding cavity, are more dynamic and exhibit more varied secondary and tertiary structures (Fig. 1). The flexibility of this architecture was first demonstrated for P450 102A1, which exhibited an open channel to the active site when crystallized without a substrate (17) and a closed form when a substrate was bound (18). Several solvent access channels (Fig. 2) that can expand, contract, and merge for substrate access and product exit have been defined from structures and molecular dynamics studies (19).

Membrane Binding

Microsomal P450s are targeted to the endoplasmic reticulum by an N-terminal leader that includes a transmembrane helix (Fig. 2) that is linked by a polar connector to the catalytic domain, which is sequestered to the cytoplasmic side of the membrane (20). With the exception of 19A1 (21), microsomal P450s have been expressed for structure determinations without their N-terminal leader sequences, as described initially for rabbit microsomal 2C5 (3, 22). The hydrophobic surfaces of helices A′, F′, and G′ of microsomal P450s provide additional interactions with the membrane surface (3, 22–25). Helices F′ and G′ are not typically seen in prokaryotic P450s, and they are formed by a longer polypeptide chain connecting helices F and G in eukaryotic membrane P450s. Helix F′ resides between
β-sheet 1 and the helix B-C loop and above a heme propionate. In membrane P450s, this heme propionate is usually positioned below the plane of the heme, which increases space below helix F’, whereas in soluble prokaryotic P450s, this propionate typically resides above the plane (26). The leader sequences targeting family 11, 24, and 27 P450s to mitochondria are cleaved upon import (27, 28), and membrane binding to the matrix side of the inner membrane is likely to reflect interactions of the hydrophobic external surfaces of helices A’ and G’ with the membrane (Fig. 2) (29, 30).

Interactions of helices A’, F’, and G’ with the membrane suggest that some substrate access channels are likely to open into the membrane, whereas those that open on sides of the active site and under the helix F-G region will open to the cytosol (Fig. 2). Molecular dynamics studies of microsomal 2C9 (31, 32) and 3A4 (26) in solution and bound to phospholipid bilayers indicate that the opening and closing of solvent channels can be modulated by such interactions compared with simulations in a homogeneous aqueous medium.

Specialist Enzymes

Mitochondrial enzymes are specialists that generate specific products that fulfill their physiologic functions. Mitochondrial 11A1 catalyzes the first step in steroid hormone synthesis by successive oxygenations that result in scission of the C21–C22 bond of cholesterol to form pregnenolone and isocaproaldehyde. Structures of 11A1 co-crystallized with cholesterol and the two intermediate products, (22R)-hydroxycholesterol and (22R,20R)-dihydroxycholesterol, bound in the active site (10) indicate that the sterol ring system is positioned under helix F’, with the side chain positioned with C22 and C20 in close proximity to the heme iron for each substrate. A struct-
tured determined for the bovine 11A1 (22R)-hydroxycholesterol complex (33) indicates that this substrate-binding site is highly conserved. Mitochondrial 24A1 catalyzes a similar reaction that cleaves the side chain of calcitriol to inactivate the hormone. A structure of rat mitochondrial 24A1 crystallized in the absence of its substrate exhibits an open substrate-binding cleft between helices A’ and F’, and when calcitriol binds, the cleft is likely to close and resemble structures of 11A1 (Fig. 2B) (30). Cholesterol 3-sulfate binds in a similar way in a structure of microsomal 46A1, with the side chain positioned for hydroxylation of C24. This is an important reaction for the clearance of excess cholesterol from the brain. Interestingly, the substrate-free structure of 46A1 exhibits a much different cavity shape (34), and the 46A1 active site adapts to bind several structurally unrelated inhibitors (35, 36). In contrast, structures of microsomal 2R1 (37) indicate that the secosterol moiety of vitamin D₃ and related compounds is bound between helices I and G and the helix B-C loop. This positions the side chain for 25-hydroxylation, which is the first step in the conversion of vitamin D₃ to calcitriol.

The sterol ring system is positioned much differently in a structure of microsomal 7A1 (Protein Data Bank code 3SN5), where cholest-4-en-3-one residues above the heme propionate side chains, with the plane of the sterol rings parallel to the heme plane. The site of metabolism, C7, is positioned closest to the heme iron. Hydroxylation at C7 is the rate-limiting step in bile acid formation from cholesterol. Similarly, a structure of the human aromatase, microsomal 19A1, crystallized with androstenedione (21) indicates that the long axis of the steroid is almost parallel to the heme plane, with the 19-methyl group positioned for reaction with the reactive intermediate. Estrogens are formed by three successive oxygenations at C19, which leads to elimination of formic acid and to aromatization of ring A (38). A structure of 19A1 complexed with exemestane, an inhibitor used clinically to reduce estrogen formation in breast cancer patients, led to the synthesis of new inhibitors with increased potency (39).

Similarly, inhibitors of microsomal 17A1 are used to inhibit androgen formation to treat prostate cancer. The first step of androgen biosynthesis is 17α-hydroxylation of pregnenolone, which is followed by a second oxygenation that results in scission of the C17–C20 bond to produce androstenedione and acetic acid. Structures of 17A1 were determined with a Food and Drug Administration-approved first-in-class inhibitor (abiraterone) and with another inhibitor (TOK-001) that is in clinical trials (40). In this case, the long axis of the inhibitors is almost perpendicular to the plane of the heme (40). A similar orientation was observed for 17α-hydroxyprogesterone in a structure of 21A2 (41). Microsomal 21A2 catalyzes the 21-hydroxylation of 17α-progesterone and progesterone to form precursors for the synthesis of cortisol by 11B1 and aldosterone by 11B2, respectively. The binding of deoxycorticosterone to mitochondrial 11B2 is similar to that of androstenedione in 19A1, but with C11 and the 18-methyl group placed near the heme iron (42).

Interestingly, human microsomal 51A1 is an anti-target for development of therapeutic inhibitors that target 51A1 in fungal pathogens. Human 51A catalyzes the 14α-demethylation of lanosterol, another carbon–carbon bond scission reaction, in the pathway for de novo synthesis of cholesterol. It is anticipated that the availability of structures for human 51A1 (43) and 51A orthologs in fungal pathogens (44–46) will aid in the design of drugs that are more selective for fungal 51A relative to the human enzyme.

**Isomerases and Other Non-monoxygenases**

Humans express two specialist microsomal P450s (8A1 and 5A1) that catalyze the isomerization of prostaglandin H₂ to produce prostacyclin and thromboxane, respectively. Structures of human (47) and zebrafish (48) 8A1 in the ligand-free state have been determined, and conserved characteristics of the active site architectures were noted (48). U51605, a substrate analog with nitrogens substituted for the endoperoxide oxygens, binds with the C11 nitrogen coordinated to the heme iron (48). This is consistent with the proposed initial binding of the C11 oxygen of the endoperoxide moiety to the heme iron to initiate the isomerase reaction (49). The C9 nitrogen exhibits a hydrogen bond with the side chain of Asn-277 on helix I of 8A1 (48). This asparagine is conserved in plant non-monoxygenases of the CYP74 family, such as chloroplast allene oxide synthase, in which the corresponding asparagine is thought to facilitate conversion of lipid peroxides to allene oxides (50). These studies also noted that alterations in the proximal surface would likely prevent interactions with electron donors for P450 monoxygenases (48, 50).

**Carcinogen-metabolizing Enzymes**

Each generalist P450 transforms a wide range of lipophilic substrates to more polar compounds to enhance elimination. Unfortunately, P450s can also transform procarcinogens to direct acting mutagens. Six microsomal P450s (1A1, 1A2, 1B1, 2E1, 3A4, and 2A6) account for >90% of known carcinogen activation pathways (51). Structures of 1A1 (Protein Data Bank code 4I8V), 1A2 (52), and 1B1 (53) co-crystallized with the inhibitor α-naphthoflavone (272 Da) indicate that their active sites are narrow, with large hydrophobic surfaces suitable for binding polynuclear aromatic hydrocarbons (Fig. 1). A bend in helix F reinforces the narrow cavities. Interestingly, amino acid residues that line these cavities are conserved in other species, which is likely to reflect persistent environmental exposure to these compounds during evolution. In contrast, sequence conservation is much less evident for portions of the polypeptide chains that form the active site surfaces of family 2 P450s. This was first predicted from sequence alignments with the primary and tertiary structures of bacterial 101A1 (54) and confirmed by structure determinations.

The active site cavity of the principal enzyme for nicotine clearance (2A6) co-crystallized with coumarin (146 Da) (55), with nicotine (162 Da) (56), and with several inhibitors (55, 57) is smaller than that of family 1 P450s. Relatively small differences in rotamer and backbone conformations are evident for these complexes. A somewhat larger and more plastic active site relative to 2A6 is evident for 2A13, which ultimately converts nicotine and cotinine to carcinogenic nitrosamines such
as nicotine-derived nitrosamine ketone (NNK\(^2\); 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane) in lung tissues (56, 58).

Microsomal 2E1 co-crystallized with indazole (118 Da) and 4-methylpyrazole (82 Da) exhibits the smallest cavity (59). The active site expands when C\(_{9r}\), C\(_{10r}\), and C\(_{12}\) fatty acids with an azole ring attached to the terminal carbon are bound. The azole group coordinates to the heme and positions the terminal aliphatic carbon of the fatty acid constituent to mimic the position for \(\omega\)-hydroxylation. A minor shift in the position of Phe-298 on helix I allows the carboxylate moieties to reside in an adjacent cavity between helices I, F, and G and increases the volume of the active site from \(\sim 190 \\text{Å}^3\) for indazole to 420–490 \(\text{Å}^3\) for the C\(_{9r}–C_{12}\) compounds (60). Structures of 2E1, 2A6, and 2A13 co-crystallized with pilocarpine reveal conformational changes and differences in binding interactions (61).

**Drug-metabolizing Enzymes**

Drug clearance pathways mediated by P450s can be a formidable barrier to the development of orally available new drugs. Structures of drug-metabolizing P450s can be used to improve predictions of sites of metabolism and provide information for drug redesign to overcome metabolic barriers to improve efficacy or to reduce the likelihood of drug-drug interactions (62–65). Hepatic clearance of drugs by P450-mediated metabolism is the most prevalent pathway for elimination of the 200 most prescribed drugs and is attributed in order of frequency to microsomal P450s 3A4, 2C9, 2D6, 2C19, 1A2, 2C8, and 2B6 (66). With the exception of 2B6, structures of these enzymes exhibit active site cavities that are larger than that of 1A2 and that are more open and pliant.

3A4 structures differ from family 1 and 2 structures because helices F and G do not extend across the active site cavity (Fig. 1). In 3A4, the “roof” above the heme is formed by a cluster of phenylalanine side chains. These phenylalanine side chains expand outward relative to the ligand-free structure (67, 68) to accommodate erythromycin (734 Da) (69), ritonavir (721 Da) (70), desthzaozolymethoxyxcarbonyl ritonavir (580 Da) (71), or two molecules of ketoconazole (531 Da) stacked antiparallel and vertically above the heme (69). In contrast, bromoenocryptine (656 Da) elicits only small changes relative to ligand-free structures when bound (72). Co-crystallization of progesterone with 3A4 identified a peripheral binding site for the steroid near the outer surface of helices F’ and G’ and the phenylalanine cluster (68). 3A4 often exhibits homo- and heterotrophic activation kinetics with progesterone and other substrates of similar size that are consistent with the binding of two or more molecules to the enzyme (73, 74). Biophysical studies have suggested that two molecules can stack in the active site (75), as seen for the 3A4 structure with two molecules of ketoconazole (69), and have provided evidence for a peripheral binding site such as that observed for progesterone (76).

The active site cavity of 2C8 is large but has a more serpentine shape than that of 3A4 (Fig. 1). The cavity readily accommodates montelukast (586 Da) or two molecules of 9-cis-retinoic acid (300 Da). The lower portion of the cavity projects from the heme iron toward the open entrance to the active site on the N-terminal side of the B-C loop under helix F’. This arm is occupied by one retinoic acid molecule, with its carboxylate in the entrance channel and the trimethylcyclohexenyl ring positioned for hydroxylation. The distal molecule is stacked above the proximal molecule, with the carboxylate in a second entrance on the C-terminal side of the B-C loop, where it forms an ionic bond with Arg-241 on helix G, and its trimethylcyclohexenyl ring is stacked above the midpoint of the proximal retinoic acid. The two arms of the cavity complement the shape of montelukast, which exhibits three large groups attached to a chiral carbon (Fig. 1). In contrast, felodipine (384 Da) and troglitazone (442 Da) occupy only a portion of the cavity (77). The structure of the enzyme crystallized in the absence of a ligand (78) does not differ greatly from that of the ligand complexes (77). This apparent rigidity could reflect the presence of a fatty acid bound to the exterior surface of 2C8, with the aliphatic chain passing through the turn formed by helices F’, G’, and G. The location of this peripheral binding site is close to that observed for progesterone in the structure of 1A2.

2C19 plays an important role in the clearance of omeprazole (345 Da) and in the conversion of clopidogrel (322 Da) to its therapeutic metabolite. The backbone conformation of 2C19 complexed with the inhibitor (2-methyl-1-benzofuran-3-yl)-(4-hydroxy-3,5-dimethylphenyl)methanone is highly similar to that of 2C8, but the cavity is divided into a smaller active site and an antechamber under helix F’, which is likely to be part of the substrate access channel. These differences between 2C8 and 2C19 arise from extensive divergence in the amino acid side chains that form the cavities of the two enzymes (79). The separation of the two cavities in 2C19 reflects a constriction formed by the close approach of phenylalanines on the B-C loop and in the turn of the C-terminal loop as seen for 2D6 (Protein Data Bank code 3QM4) (Fig. 3A). This antechamber is not evident in P450s 1A, 1B, 2A, and 2E because this region is filled by larger amino acid side chains.

Similarly, the antechamber is reduced in volume in rabbit 2C5 because the differences in the conformation of the F-F’ loop fill this space as they do in family 1 P450s (Fig. 1) and in the substrate-free 2D6 structure (Protein Data Bank code 2F9Q) (Fig. 3). Nevertheless, the corresponding phenylalanines exhibit significant rotamer differences as well as changes in the F-G region, the C-terminal loop, and the B-C loop when different substrates are bound (80, 81).

Although 2C9 exhibits >90% sequence identity to 2C19, the two enzymes make unique contributions to human drug metabolism. 2C9 contributes extensively to the clearance of small anionic compounds, and 2C9 genetic variation increases risks for adverse effects of warfarin and phenytoin therapies. The functional differences between 2C9 and 2C19 arise from conformational differences due to amino acid differences that reside outside the active site cavity but influence the architecture of the 2C9 active site (79). The alternative conformation of the 2C9 B-C loop positions the conserved Arg-108 in the active site, where it forms an ionic bond with the carboxylate of flurbiprofen in the 2C9 structure (Protein Data Bank code 1R9O). This rearrangement alters both the polar properties and the shape of the active site cavity and underlies the role of 2C9 in the metabolic clearance of other small anionic drugs and the

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\(^2\) The abbreviation used is: NNK, nicotine-derived nitrosamine ketone.
distinct contributions of the two enzymes to drug metabolism (79, 82).

Two additional 2C9 structures (Protein Data Bank codes 1OG2 and 1OG5) determined for a construct with seven amino acid substitutions in the helix F-G region to facilitate crystallization (83) exhibit much larger active site cavities than seen for 2C9 (code 1R9O) (82) or for 2C19 (79). This difference reflects an elevation of the helix F-G region above the heme, a repositioning of the turn in the C-terminal loop away from helix I, and an alternative conformation of the helix B-C loop that places Arg-108 outside the active site in the 1OG2 and 1OG5 structures. S-Warfarin (308 Da) is bound in a distal portion of the 1OG5 structure of the 2C9 mutant, which was speculated to serve as an effector site or possibly initial binding site (83). This location corresponds to the antechamber seen in 2C19, and the warfarin-binding site could reflect an initial binding site in the entry way. A similar antechamber binds a second substrate molecule in the structure of 21A2 (41). Similarly, one of two molecules of NNK (207 Da) occupies a similar position in an expanded 2A13 active site, with the other NNK positioned near the heme (56). In contrast, a 2A13 structure for an alternative crystal form exhibits one molecule of NNK positioned for metabolite formation in a small active site for six of eight molecules in the asymmetric unit. Interestingly, one of the two remaining 2A13 molecules of the asymmetric unit exhibits an open access channel under helix F and between the C-terminal loop and helix I (56). This channel is also open in the 2F9Q structure of 2D6 (Fig. 3A) crystallized in the absence of a ligand (84).

In contrast, the 3QM4 structure of 2D6 complexed with prinomastat (423 Da) exhibits a closed active site cavity that conforms closely to the size of prinomastat (85). Additionally, an antechamber is evident in the structure of the prinomastat complex below helix F’, similar to that seen in 2C19 (79) and 21A2 (41). There is a significant difference in the conformation of the helix F-G region and neighboring portions of the two structures, and the antechamber is filled in the 2F9Q structure of 2D6 due to the alternative conformation of the helix F-G region (Fig. 3B).

A second open structure of 2D6 (Protein Data Bank code 3TBG) was crystallized with thioridazine (371 Da) in the active site, where it forms an ionic bond with Asp-301 on helix I. Many substrates and inhibitors of P450 2D6 have basic nitrogens that are positively charged at neutral pH and that are thought to bind to either Asp-301 or Glu-216 in the active site (86). A second thioridazine is bound in the antechamber of the 3TBG structure. The constriction between the active site cavity and the antechamber of the 3QM4 structure is relaxed to form a continuous channel encompassing the two thioridazine molecules that extends from the active site to the open entrance between the helix F’ and the helix A’ regions in the 3TBG structure (Fig. 3A). The thioridazine in the entrance channel forms an ionic bond with Glu-222 on helix F’, suggesting that Glu-222 could facilitate the initial binding of cationic substrates to 2D6. Although binding of a second molecule in the entrance channel could act as an effector, biochemical evidence to support this notion has not been reported for 2D6 with thioridazine, 2A13 with NNK, or 21A2 with 17α-hydroxyprogesterone at enzyme...
and substrate concentrations used to assess catalytic activity or ligand binding, which are much lower than those used for crystallization.

The structural flexibility required for opening and closing the cavity can also enable the binding of larger inhibitors or alternative substrates in more open structural conformations. This has been studied extensively in rabbit 2B4, which was first crystallized in an open ligand-free form (87) and subsequently in closed structures with 4-(4-chlorophenyl)imidazole (179 Da) (88) and 1-(4-chlorophenyl)imidazole (179 Da) (89) bound in a small active site cavity. A structure of human 2B6 exhibits a remarkably similar active site when crystallized with 4-(4-chlorophenyl)imidazole (90). Additionally, ticlopidine (264 Da) and clopidogrel (322 Da) complexes of 2B4 exhibit compact closed structures (91), and more recently, ligand-free 2B4 was crystallized in a closed conformation (92). Similarly, complexes of 2B6 with 4-benzylypyridine (169 Da) and 4-(4-nitrobenzyl)pyridine (214 Da) are closed, and this is accompanied by small adaptations (93). Systematic use of azole ligands of increasing size with 2B4, 1-biphenyl-4-methyl-1H-imidazole (234 Da) (94) and bifenazole (310 Da) (95), produced a continuum of structures from small and closed to larger and more open active site cavities (96). 2B6 and 2B4 have also been crystallized with two molecules of amlodipine (409 Da) in an expanded active site channel (97).

Additionally, structures have been determined for covalent adducts of 2B4 with reactive metabolites of the irreversible inhibitors 9-ethylphenanthrene (202 Da) (98) and tert-butylphenylacetylene (176 Da) (99) covalently linked to Thr-302 by an ester bond. The latter was crystallized in both the closed and open conformations. An alternative rotamer of the adducted residue opens the active site sufficiently to allow access of other substrates and may underlie the residual activity of the enzyme following addition. Similarly, double occupancy of open conformations of P450s could underlie substrate-dependent differences in $K_i$ values observed for drug-drug interactions.

**Perspective**

Structures determined for specialist P450s reveal adaptations that underlie their unique physiologic roles, which require precise positioning of the substrate to produce the appropriate product. It is likely that structural characterization of human P450s will be extended to include P450s in other physiologic pathways as well as P450s targeted for prodrug activation in tumors. Additionally, structural characterization of membrane P450s in other species, including plants and insects, would increase our understanding of the mechanisms of resistance to P450s in other physiologic systems (93), and more recently, ligand-free 2B4 was crystallized in a closed conformation (92). Similarly, complexes of 2B6 with 4-benzylypyridine (169 Da) and 4-(4-nitrobenzyl)pyridine (214 Da) are closed, and this is accompanied by small adaptations (93). Systematic use of azole ligands of increasing size with 2B4, 1-biphenyl-4-methyl-1H-imidazole (234 Da) (94) and bifenazole (310 Da) (95), produced a continuum of structures from small and closed to larger and more open active site cavities (96). 2B6 and 2B4 have also been crystallized with two molecules of amlodipine (409 Da) in an expanded active site channel (97).

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