The Crystal Structure of Cytochrome P450 4B1 (CYP4B1) Monooxygenase Complexed with Octane Discloses Several Structural Adaptations for ω-Hydroxylation

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P450 family 4 fatty acid ω-hydroxylases preferentially oxygenate primary C–H bonds over adjacent, energetically favored secondary C–H bonds, but the mechanism explaining this intriguing preference is unclear. To this end, the structure of rabbit P450 4B1 complexed with its substrate octane was determined by X-ray crystallography to define features of the active site that contribute to a preference for ω-hydroxylation. The structure indicated that octane is bound in a narrow active-site cavity that limits access of the secondary C–H bond to the reactive intermediate. A highly conserved sequence motif on helix I contributes to positioning the terminal carbon of octane for ω-hydroxylation. Glu-310 of this motif auto-catalytically forms an ester bond with the heme 5-methyl, and the immobilized Glu-310 contributes to substrate positioning. The preference for ω-hydroxylation was decreased in an E310A mutant having a shorter side chain, but the overall rates of metabolism were retained. E310D and E310Q substitutions having longer side chains exhibit lower overall rates, likely due to higher conformational entropy for these residues, but they retained high preferences for octane ω-hydroxylation. Sequence comparisons indicated that active-site residues constraining octane for ω-hydroxylation are conserved in family 4 P450s. Moreover, the heme 7-propionate is positioned in the active site and provides additional restraints on substrate binding. In conclusion, P450 4B1 exhibits structural adaptations for ω-hydroxylation that include changes in the conformation of the heme and changes in a highly conserved helix I motif that is associated with selective oxygenation of unactivated primary C–H bonds.

Cytochrome P450 family 4 fatty acid ω-hydroxylases are heme-containing monooxygenases that provide pathways for the reduction of potentially toxic non-esterified fatty acids and for the elimination of excess nutritive and non-nutritive lipids by catalyzing the addition of oxygen to a C–H bond of the terminal ω-carbon of fatty acids (1, 2). The resulting ω-alcohols are generally oxidized further to produce dicarboxylic acids, but they also may be transformed to glucuronides or esterified to glycerolipids. Urinary dicarboxylic fatty acids are elevated in humans by fasting or in uncontrolled diabetes, which are conditions where adipocyte lipolysis increases the availability of non-esterified fatty acids to fuel metabolism (3). It is estimated that roughly 15% of fatty acids undergo ω-hydroxylation during peak periods of fatty acid catabolism (4), whereas this fraction is much smaller under conditions where concentrations of non-esterified fatty acids are low (5). Collectively, these enzymes provide pathways for the metabolic clearance of branched chain fatty acids, eicosanoids, and xenobiotic substrates such as dietary phytanic acid, drugs, toxins, and vitamins E and K (1, 2, 6).

Similar to other P4502 gene families encoding multipurpose enzymes for the elimination of xenobiotic compounds, family 4 exhibits genetic diversity between species. Twelve of the 57 cytochrome P450 genes in the human genome encode family 4 P450s, which are assigned to six subfamilies in mammals designated by a letter. P450s 4B1, 4V2, and 4X1 have orthologues in other vertebrate species, whereas the P450 4A and 4F subfamilies exhibit paralogues with differences in the number of genes for these two subfamilies in other species (1, 2). Although these enzymes often exhibit overlapping substrate specificities, genetic association studies link the ω-hydroxylase CYP4V2 to the Bietti’s crystalline dystrophy, which is characterized by ocular lipid deposits (7, 8), suggesting a key role for CYP4V2 in the removal of excess lipids in ocular tissues. Furthermore, CYP4F22 genetic variants are associated with lamellar ichthyosis, which reflects deficiencies in the water permeability barrier of skin (9). This is likely to reflect the capacity of P450 4F22 to catalyze the ω-hydroxylation of very long chain fatty acids (C28 or greater). The resulting ω-hydroxylated very long chain fatty acids are essential for the formation of acylceramides, which are components of the water permeability layer of the

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‡ This article contains supplemental Fig. S1 and supplemental File P450-4A11-homology-model.pdb. The atomic coordinates and structure factors (code 5T6Q) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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2 The abbreviations used are: P450, a generic term for a cytochrome P450 enzyme; individual P450s are identified using a number-letter-number format based on amino acid sequence relatedness; PDB, Protein Data Bank; SSRL, Stanford Synchrotron Radiation Lightsource; TMH, transmembrane helix; Fw, observed structure factor; Fc, calculated structure factor; IPTG, isopropyl 1-thio-β-D-galactopyranoside.
skin. Additionally, some cytochrome P450 ω-hydroxylases, such as human cytochrome P450s 4A11, 4F2, and 4F5b, catalyze the formation of 20-hydroxyeicosatetraenoic acid from arachidonic acid, which contributes to regulation of vascular tone and renal sodium absorption (10, 11). Genetic association studies link variants of human CYP4A11 and CYP4F2 with increased risks for hypertension, which in the case of CYP4A11 is likely to reflect dysregulation of renal sodium uptake as the blood pressure increase is sensitive to levels of dietary salt (12, 13).

Fatty acid ω-hydroxylation reflects the addition of oxygen to a primary C–H bond of the terminal carbon of the fatty acid. This reaction is generally considered to be the most difficult reaction catalyzed by cytochrome P450 monoxygenases because the enzyme needs to exclude access of neighboring and more reactive secondary C–H bonds to the iron-bound reactive oxygen intermediate. The reactive intermediate is formed by the reduction of oxygen bound to the heme iron followed by protonation and heterolytic scission of the dioxygen bond with elimination of the water molecule and formation of a highly electrophilic oxygen atom bound to the hypervalent heme iron. Oxygenation of aliphatic C–H bonds is thought to proceed by abstraction of a hydrogen by the iron oxo intermediate followed by recombination of the resulting substrate radical with the transiently iron-bound hydroxyl radical (14). As the strength of primary C–H bonds is greater than that of secondary C–H bonds, oxygen addition at the ω-1 or ω-2 positions of fatty acids is typically seen for less specialized enzymes (15–17). These considerations suggest that the ω-hydroxylases have structural features that favor the approach to the reactive iron oxo species of the ω-carbon relative to the ω-1 carbon (18).

Within human family 4 cytochrome P450 enzymes, the capacity for ω-hydroxylation is associated with a substitution of glutamic acid for the first alanine or glycine in the canonical sequence motif, (A/G)Gx(D/E)T (6). This sequence motif is located on helix I in the active site near the heme iron. The presence of the glutamic acid near the heme iron leads to autocatalytic formation of an ester bond between the glutamic acid and the 5-methyl group of the heme (19–21). This covalent bond, oxygen addition at the ω-1 or ω-2 positions of fatty acids is typically seen for less specialized enzymes (15–17). These considerations suggest that the ω-hydroxylases have structural features that favor the approach to the reactive iron oxo species of the ω-carbon relative to the ω-1 carbon (18).

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The isolated protein was crystallized in the presence of octane, and the structure of the P450 4B1-octane complex was determined using X-ray diffraction data collected from a single crystal at 100 K on Stanford Synchrotron Radiation Lightsource (SSRL) beam line 12-2. Initial phasing was obtained by molecular replacement using Phaser (33) as implemented in Phenix. This search identified the enantiomeric space group as P 21 21 2 with a single protein chain in the asymmetric unit. P450 4B1 (an enzyme that hydroxylates the alkyl side chain of cholesterol) is the most closely related and structurally characterized P450 by sequence similarity. Although the sequence identity was low (<30% identity), detailed 2mFo − DFo and mFo − DFo maps provided evidence for the covalently bound heme prosthetic group, the presence of the substrate octane, and sequence-related side-chain differences. The Auto-build module in Phenix was able to generate an initial model with side chains for much of the core helix bundle as well as backbone traces for other regions of regular secondary structure. The final model was built and refined iteratively against the data to a limiting resolution of 2.70 Å using Coot (35) and Phenix, respectively. The model encompasses residues 20–501 of the protein (Fig. 2A). Residue numbers in the structure correspond to that of the native protein (UniProt, P15128). The five remaining residues of the native protein and the His tag at the C terminus as well as the two amino acids added at the N terminus to facilitate purification and initiate mRNA translation, respectively, could not be modeled. Additionally, residues 196–200 in the connector between helices E and F and residues 272–276 in the connector between helices G and H could not be modeled as these surface loops were not well defined by the electron density maps. The overall fit of the model to the data and quality assessment statistics are documented in Table 1. Additional validation results are available from the PDB, code 5T6Q.

Two cysteine pairs were identified that could potentially form disulfide bonds. One pair, Cys-364 (helix K) and Cys-336....

**FIGURE 1.** Octane shifts the Soret absorption band of CYP4B1 to lower wavelengths. A, maximum absorption for the Soret band of CYP4B1 (red) exhibits a wavelength of 419 nm indicative of a predominantly low spin ferric heme. Incremental increases in the concentration of octane of 0.5 (orange), 1 (yellow), 2 (green), 4 (cyan), 8 (blue), 16 (dark blue), and 32 μM (violet) shift the Soret maximum to lower wavelengths indicative of a high spin ferric heme. B, concentration dependence of the difference in absorption was fit to the quadratic form of the one-site binding equation by non-linear regression and a protein concentration of 0.90 μM as described under “Experimental Procedures.” The estimated dissociation constant is 0.32 μM with a maximum absorbance change of 0.069 for the experiment shown. The mean and standard error for values of the dissociation constant obtained from five replicate experiments is 0.34 ± 0.10 μM.

**FIGURE 2.** A, secondary and tertiary structure of rabbit cytochrome P450 4B1. The truncated N-terminal TMH domain is colored dark gray, the polar linker region is colored light gray, and the catalytic domain is colored from red beginning with residue 42 to blue at the C terminus. The heme prosthetic group is depicted as a stick figure with the iron shown as a sphere. The semi-transparent tubes illustrate tunnels rendered by Mole leading from the active-site cavity above the heme to the surface of the enzyme. B, linker region and TMH form a continuous A’ helix. Polar interactions between the linker region and the catalytic domain are depicted by dashed lines between the labeled amino acid residues shown as stick figures. Nitrogen, oxygen, and iron atoms are colored blue, red, and orange, respectively. C, trajectory of the A’ helix relative to the catalytic domain is compared with that of human P450 3A4 (PDB code 1TQN) and P450 51A1 from S. cerevisiae (PDB code 4LXJ). The 51A1 TMH is colored dark green; the relatively short polar linker is lime green, and a portion of the catalytic domain is depicted in pale green. Helix A’ in the linker region of 3A4 is colored copper, and a portion of the 3A4 catalytic domain is colored pale yellow.

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TABLE 1
Data reduction and structural refinement statistics

| Data reduction          | Model refinement            | PDB code 5T6Q |
|------------------------|-----------------------------|---------------|
| Space group            | Resolution (Å)              | 38.37–2.70 (2.81–2.70) |
| P 3 2 1                | Unique reflections          | 24,666 (2695) |
| a, b, c (Å)            | Test set (%)                | 4.88          |
| 109.68, 109.68, 125.82 | R work (%)                  | 20.01 (26.80) |
| α, β, γ (°)            | R test (%)                  | 24.02 (30.16) |
| 90, 90, 120            | Heavy atoms                 | 3932          |
| Resolution (Å)         | Root mean square deviation  | 0.008 Å       |
| 38.37–2.70 (2.83–2.70) | bond lengths                | 0.846°        |
| Unique reflections      | Root mean square deviation  |               |
| 24,603 (3210)          | bond angles                 |               |
| R merge                 | Ramachandran plot           | MolProbity    |
| 0.056 (0.504)          | Favored (%)                 | 96.6          |
| CCl/2 (%)              | Allowed (%)                 | 3.2           |
| 99.9 (87.6)            | Outliers (%)                | 0.2           |
| Mean (一个重要 clinical term) (SD/| Mean B-factors (Å²)         |               |
| 20.2 (4.0)             | Protein                     | 62.8          |
| Completeness (%)       | Heme                        | 42.9          |
| 99.8 (98.9)            | Octane                      | 45.3          |
| Multiplicity           | Solvent                     | 49.8          |
| 7.1 (7.2)              |                               |               |
| Wilson B-factor (Å²)   |                               |               |
| 62.7                   |                               |               |
| Protein chains in ASU  |                               |               |
| 1                      |                               |               |

ASU is asymmetric unit.

Structural Overview—The tertiary structure and major elements of secondary structure of the P450 4B1 catalytic domain resemble that of other mammalian microsomal P450s. The distal surface of the catalytic domain from the heme prosthetic group of the enzyme is shown in Fig. 2A. The polypeptide chain of the catalytic domain is traced from red to blue beginning with proline 42. Conserved secondary structures are labeled according to commonly observed conventions for P450s (39, 40). The heme covalent binding is associated with an out-of-plane distortion of the 5-methyl group and the attached heme pyrrole (helix J), with the sulfur atoms separated by 3.6 Å is conserved in other family 4 enzymes. A second pair, Cys-368 (helix K) and Cys-325 (helix I), with their sulfur atoms separated by 4.0 Å is not conserved in family 4B. These distances are too large to reflect covalent bonding between the two pairs of sulfur atoms. As synchrotron radiation can reduce disulfide bonds (36), it is possible that one or both pairs were bonded in the protein prior to data collection.

Covalent Linkage of the Heme and Glu-310—The heme cofactor is the catalytic center of the protein, and in P450 4B1, the 5-methyl group of the heme is linked covalently to protein by an ester bond with Glu-310 that forms auto-catalytically (21). The ester bond is clearly evident as defined by continuous electron density shown in Fig. 3A, which displays the refined structure with an unbiased 2mFo − DFo electron density omit map computed without the heme and Glu-310 in the phasing model used for calculation of the map. Although it was reported previously that P450 4B1 expressed in E. coli might incorporate 40% of the heme in an alternative conformation that placed the 8-methyl group near Glu-310 (41), there was no evidence for this in the crystallized protein based on well defined density for the location of the vinyl groups that define the asymmetry of the heme and the absence of additional electron density for the alternative conformation of the heme.

The covalent binding is associated with an out-of-plane distortion of the 5-methyl group and the attached heme pyrrole.
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FIGURE 3. A, omit \(2m_F - DF\) electron density map contoured at 1.5 \(\sigma\) (gray mesh) and phased without Glu-310 and the heme define the covalent linkage of the heme to Glu-310. \(B\), out-of-plane distortion of the 5-methyl and attached pyrrole relative to the plane of the heme macrocycle and the orientation of the heme 7-propionate into the active site. \(C\), omit \(2m_F - DF\) electron density map contoured at 1.5 \(\sigma\) (gray mesh) and phased without octane defines the position of octane in the active site. \(D\), hydrogen bonding and ionic interactions (dashed lines) that contribute to the observed position of the heme 7-propionate. The red and orange spheres represent a water molecule and the heme iron, respectively. Octane carbons are colored slate, and sulfur is colored yellow. Colors for other atoms correspond to those in Fig. 2A.

Relative to plane of the macrocyclic ring of the heme (Fig. 3B). Mammalian peroxidases exhibit similar covalent linkages between acidic side chains of the peroxidases and the 5-methyl and 8-methyl carbons of their heme prosthetic groups, and similar out-of-plane distortions of their heme prosthetic groups are evident in structures of these enzymes (42). Resonance Raman studies suggest that the out-of-plane distortions of the heme in lactoperoxidase are relaxed when the enzyme is cleaved by limited proteolysis suggesting that protein interactions with the heme in the enzyme’s active site induce the distortion (43).

For the final refinement of the model, a semi-empirical quantum mechanical approach was employed to minimize the Glu-310/heme ester geometry in conjunction with maximizing of the fit with the X-ray diffraction data using the DivCon 6.1 module with Phenix 1.10 (44). The PM6 Hamiltonian, which includes optimized parameters for the iron, was used for this purpose. The main advantage of this approach is optimization of the bond lengths and angles that reflect the formation of the ester bond and the effects of out-of-plane deformations on electron delocalization in the macrocyclic structure of the heme. When compared with a starting structure refined with the conventional heme restraint file, refinement using the DivCon plugin with Phenix reduced the computed strain energy relative to the starting model and improved the fit to the electron density based on real space residuals and real space correlation coefficients.

Additionally, the electron density maps (Fig. 3A) indicated that the orientation of the heme 7-propionate was on the substrate side of the macrocyclic ring of the heme where it interacts with octane (Fig. 3, C and D) as it approaches the heme iron. Although this orientation of the 7-propionate is commonly seen in structures of prokaryotic P450s, it is unusual for structures of mammalian microsomal P450s (45). Other mammalian P450 monooxygenases that exhibit this 7-propionate orientation are CYP51A1 (the sterol 14α-demethylase) and CYP46A1, and a similar heme 7-propionate interaction with a tyrosine is evident in structures of CYP51A1 and CYP46A1. In contrast, the heme 7-propionate in structures of most mammalian microsomal P450s is oriented on the other side of the heme plane (45), where it interacts with basic residues corresponding to Arg-380 of 4B1 (Fig. 3D) that resides on the strand of \(\beta\)-sheet 1 nearest to the heme. In the structure of 4B1, there is a bridging water molecule between the 7-propionate and Arg-380. The water molecule exhibits additional hydrogen bonding interactions with the side-chain hydroxyl group of Ser-442 and the carbonyl oxygen of Arg-446. This arginine also exhibits hydrogen bond stabilized ionic interactions with the heme 6-propionate. The heme 7-propionate exhibits additional H-bonds with Lys-105 and Tyr-110. Tyr-110 together with the heme 7-propionate form part of the surface of the active-site cavity that contributes to positioning of the substrate relative to the heme iron (Figs. 3 and 4).

Substrate-binding Site—Electron density maps indicated that a molecule of octane was bound in the active site with a terminal carbon close to the heme iron (3.4 Å) (Fig. 3C). The active-site cavity of P450 4B1 is highly optimized for \(\omega\)-hydroxylation of aliphatic substrates by providing a hydrophobic cavity that constrains the linear \(n\)-octane in a plane. As indicated earlier, hydrogen abstraction is generally disfavored for primary C–H bonds due to their higher bond strengths relative to adjacent secondary or tertiary C–H bonds or sites where functionalization reduces the bond strength, e.g. benzyl groups or allylic carbons (15–17). As a consequence, P450 4B1 must restrict access of the adjacent secondary C–H bonds to reactive intermediate to display a preference for hydroxylation of the primary C–H bond in P450 4B1 (46) as well as in other \(\omega\)-hydroxylases (18).

The active-site cavity of the P450 4B1 octane complex exhibits a slot-like active site that is narrow near the heme iron and broadens near the solvent channel that exits the active site between Gln-218, Gln-377, Tyr-379, and Pro-376 (Fig. 4A). The narrowest aspect of the cavity largely constrains octane in a plane. The depth of the slot is greater and accommodates the alternating up and down orientation of the carbon atoms along the aliphatic chain (Fig. 4B) until the active-site cavity broadens near the solvent channel to the surface of the protein. Branched substrates such as 4-methylheptane, valproic acid, and other short carboxylic acids (46) are likely to be accommodated in this narrow cavity without significant changes in the protein. As a result of these narrow constraints, the primary C–H bonds are more accessible to the reactive oxygen than the secondary C–H bonds of the C2 and C3 carbons. As shown in Fig. 4C, the amino acid residues lining the active-site cavity as well as adjacent residues of rabbit P450 4B1 are highly conserved for other characterized mammalian P450 4B1’s, in sharp contrast to the high degree of active site divergence evident for family 2 paralogues that contribute to a broad capacity for xenobiotic metabolism (47). Moreover, the residues that position the substrate near the heme iron (labeled with bold text in Fig. 4, A and B) are highly conserved in human \(\omega\)-hydroxylases (Fig. 5).
The importance of the Glu-310 ester bond with the 5-methyl of the heme can be seen as both the C$^\beta$/H9252 and C$^\gamma$/H9253 carbons of Glu-310 contact the octane molecule, and when oxygen binds to the iron, octane will need to move outward from 3.14 to $4 \, \text{Å}$ from the iron to accommodate it. The presence of the Glu-310 C$^\gamma$/H9253 contact will provide an increasing critical restraint for positioning a terminal C–H bond near the reactive iron at the larger distance (Fig. 4, D and B). Such a shift is likely to accommodate the binding of molecular oxygen to the heme iron during the catalytic cycle. Additionally, the ester bond formed by Glu-310 stabilizes this interaction because of increased rigidity of the side chain in relation to the heme prosthetic group, which prevents alternative conformations of the Glu-310 side chain that might conflict with substrate binding in the active site. Moreover, the formation of the ester bond creates a less polar active site for the binding of the hydrocarbon. In contrast, the charged non-esterified glutamate might stabilize the binding of water in the active site or interact directly with the heme iron to compete with substrate binding as seen in a structure of the P450 BM3 A264E mutant (48). As Glu-310 is tethered to the heme, no unfavorable loss of Glu-310 side-chain conformational entropy is associated with substrate binding.

In addition to the glutamic acid residue 310, flanking residues on helix I are also conserved in human P450 family 4 $\omega$-hydroxylases (6), as illustrated in Fig. 5, as well as in other family 4 $\omega$-hydroxylases (6).
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P450s in a variety of species (19), although this motif is less conserved in family members that catalyze other reactions, such as human P450s 4F12, 4F8, 4X1, and 4Z1 that do not have a glutamic acid residue corresponding to Glu-310 of 4B1. Two of these residues compensate for hydrogen bonds that are lost due to extension of the turn in helix I adjacent to the Glu-310. Conserved His-312 donates an H-bond to the carbonyl of Met-308 (Fig. 3, A and C). Similarly, the side-chain hydroxyl group of conserved Thr-314 forms an H-bond with the Glu-310 carbonyl oxygen in the peptide bond with Gly-311 (Fig. 3, A and B).

A third conserved residue, Phe-309, is oriented to cap the height of the cavity above the heme iron and the terminal carbon of octane. The cap over the substrate-binding cavity is propagated by the close packing of the Leu-485 (Fig. 4, dark blue carbons) on the turn in β-sheet 4 of the C-terminal loop (Fig. 2A). Both residues are conserved in the human family 4 ω-hydroxylases. This constraint on the substrate is reinforced by a secondary layer of bulky amino acid side chains on helices B’, F, and G. Another conserved residue in the human ω-hydroxylases, Val-375, on the connector between helix K and β-sheet 1–3 (Fig. 4, cyan carbons) fills the space under the C-terminal loop and contacts Thr-314 to form the side of the cavity opposite Glu-310 (Fig. 4A). An additional residue conserved in the human ω-hydroxylases, Leu-122 (yellow carbons) on the β-turn between helices B’ and C, fills the space between Glu-310 and Tyr-110 (Fig. 4, yellow-orange carbons). Together, these highly conserved residues form the narrow cavity for the approach of octane to the heme iron (Fig. 4). In the human family 4 enzymes that lack the glutamic acid corresponding to Glu-310, these additional active-site residues are less well conserved (Fig. 5) indicating that the catalytic divergence of these enzymes involves additional changes near the catalytic center of the active site. Although the substrate profiles for human ω-hydroxylases differ, these comparisons suggest that the human ω-hydroxylases exhibit very similar features for channeling the aliphatic ends of their substrates to the reactive oxygen as would be expected to achieve a preference for oxygenation of the primary C–H bonds.

Effects of Amino Acid Substitutions for Glu-310—The effects of E310A, E310D, and E310Q substitutions on the regioselectivity of 4B1 catalysis for ω and ω-1 hydroxylation of heptane, octane, nonane, and decane were determined using reconstituted full-length enzymes (Fig. 6 and Table 2). Of the three mutants, the E310A mutant exhibited the highest rates of total metabolite formation but with a reduction in the preference of ω relative to ω-1 hydroxylation. Nevertheless, a preference for ω-hydroxylation by the E310A enzyme was retained for n-heptane and n-octane but not for n-nonane and n-decane, which is consistent with an earlier report that substitution of an alanine for Glu-310 in 4B1 reduces the preference for ω-hydroxylation of lauric acid relative to hydroxylation of adjacent secondary C–H bonds (21). The retention of ω-hydroxylase activity by the E310A enzyme for the shortest chain length substrates is likely to reflect other conserved features of the active site that restraints substrates near the heme iron in the absence of the conserved glutamic acid and compensates for changes related to the loss of the covalent linkage of the heme to the enzyme. The E310D and E310Q mutants have longer side chains than the E310A mutant, which favors retention of the preference for ω-hydroxylation albeit with lower rates. This suggests that when the n-alkane is bound in a position for hydroxylation, the aspartate and glutamine side chains adopt a similar orientation as the ester-linked side chain of Glu-310 within the crowded active-site cavity. In the case of the aspartate substitution, the autocatalytic formation of the 5-methyl radical intermediate is likely to occur based on conversion of the 5-methyl to a 5-hydroxymethyl group, but the shorter reach of the aspartate does not permit formation of the ester. Isotope enrichment studies indicate that the proposed 5-methyl radical intermediate reacts with water to form a 5-hydroxymethyl group in the E310D mutant (49). Although the aspartate and glutamine side chains cannot form the ester bond, their greater conformational entropies relative to the tethered glutamate are likely to reduce the rate of reaction even though they restrain the substrate for ω-hydroxylation.

As reported previously for the wild-type enzyme expressed in insect cells, the ratio of ω- to ω-1 hydroxylation diminishes with the increase in substrate chain length from heptane (23-fold) to decane (1.6-fold), as does the total rate of metabolite formation (46). Similar values were obtained with the reconstituted full-length 4B1 expressed and purified from E. coli (Table 2 and Fig. 6). This dependence of rates of formation on substrate chain length is also evident for the E310A, E310D, and E310Q mutants, although n-heptane is metabolized at lower rates than n-octane by these mutants. As seen in Fig. 4, octane almost completely fills the substrate-binding cavity suggesting that the binding of longer fatty acids and n-alkanes might effect changes in the cavity size. Consistent with this notion, the rates of hydroxylation and the ratio of ω to ω-1 hydroxylation decreased for n-alkanes with increasing chain length. Additionally, the rates for fatty acid hydroxylation are typically lower than for the corresponding n-alkane, and the ratios of ω to ω-1 hydroxylation are lower (46). As the active site of the octane complex does not contain a basic residue to complement a negatively charged carboxylate moiety of fatty acids, the enzyme may
adopt a more open form with greater hydration of the entrance channel to facilitate binding of the fatty acid substrates as seen in P450 2C8 (50).

Overall, results of these mutagenesis studies are consistent with optimization of the 4B1 active-site cavity for the shorter hydrocarbons, and the binding of the longer compounds may force changes in the cavity to accommodate them albeit with less energetically favored binding modes and increased access of the secondary C−H bonds to the reactive intermediate. As shown in Fig. 4C, the residues that form this active-site cavity are highly conserved in family 4B1 enzymes from other mammalian species. The residues that form the distal portion of the substrate-binding cavity diverge more extensively (Fig. 5) and potentially underlie differences in substrate profiles for other family 4 enzymes in other subfamilies when compared with 4B1. Phe-113, which impinges on the substrate-binding cavity are likely to be retained to facilitate ω-hydroxylation.

In contrast, the sequence divergence in the distal portions of the substrate-binding cavity are likely to underlie distinct substrate profiles exhibited among family 4 ω-hydroxylases.

**Experimental Procedures**

**Materials and Reagents**—The following chemicals were purchased from Sigma: δ-aminolevulinic acid, sodium cholate, L-histidine, DNase I, PMSF, and octane. IPTG and HEGA 8 were obtained from Anatrace (Maumee, OH); lysozyme was from Worthington; nickel-nitrilotriacetic acid-agarose was from Qiagen (Valencia, CA), and HA Ultragel® was obtained from PALL Life Sciences (Port Washington, NY). Pierce protease inhibitor tablets (EDTA-free) and all other chemicals were purchased from Thermo Fisher Scientific.

Expression and Purification of Truncated Rabbit P450 4B1 for Crystallization—E. coli strain DH5α transformed with pCW-4B1 #7 (28) was used for protein expression. Single colonies were grown in super broth at 37 °C overnight. The bacterial culture was pelleted at 2500 × g for 10 min and then suspended in Terrific Broth. The culture media were shaken at 200 rpm at 37 °C until the A600 reached 0.4−0.5. After 30 min of incubation at 27 °C at 120 rpm, the 4B1 protein expression was induced by addition of 1 mM IPTG and 0.5 mM δ-aminolevulinic acid. Cells were harvested after 44−48 h. The expression level was routinely ~1200 nmol of P450 per liter of bacterial culture.

Truncated P450 4B1 was purified using nickel-nitrilotriacetic acid-agarose followed by hydroxylapatite chromatography as described (28) with modifications. Briefly, the harvested cells were suspended in 20 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol (v/v), and protease inhibitors (0.5 mM PMSF and EDTA-free Pierce protease inhibitor tablets

| Substrate | P450 4B1 | ω | ω-1 | ω-2 | Total |
|-----------|---------|---|----|----|------|
| n-Heptane | WT      | 24.7 ± 2.1 | 1.13 ± 0.17 | 0.02 ± 0.01 | 25.8 ± 2.0 | 22.3 ± 4.6 |
|           | E310A   | 14.1 ± 0.5 | 2.00 ± 0.07 | 0.14 ± 0.01 | 2.14 ± 0.06 | 7.03 ± 0.23 |
|           | E310D   | 2.52 ± 0.19 | 0.04 ± 0.00 | 0.03 ± 0.03 | 0.58 ± 0.03 | 65.7 ± 1.0 |
|           | E310Q   | 1.79 ± 0.21 | 0.03 ± 0.01 | 0.00 ± 0.00 | 0.82 ± 0.21 | 54.3 ± 5.7 |
| n-Octane  | WT      | 18.4 ± 2.3 | 2.56 ± 0.39 | 0.02 ± 0.01 | 21.0 ± 2.7 | 7.21 ± 0.18 |
|           | E310A   | 12.7 ± 0.6 | 7.26 ± 1.20 | 0.15 ± 0.01 | 20.1 ± 1.7 | 1.78 ± 0.27 |
|           | E310D   | 7.05 ± 0.57 | 0.26 ± 0.13 | 0.01 ± 0.01 | 7.30 ± 0.68 | 32.9 ± 18.8 |
|           | E310Q   | 4.16 ± 0.21 | 0.24 ± 0.02 | 0.01 ± 0.01 | 4.41 ± 0.23 | 17.2 ± 0.5 |
| n-Decane  | WT      | 8.93 ± 0.69 | 3.68 ± 0.20 | 0.05 ± 0.01 | 12.7 ± 0.9 | 2.43 ± 0.12 |
|           | E310A   | 5.27 ± 0.11 | 8.74 ± 0.27 | 0.35 ± 0.02 | 14.4 ± 0.4 | 0.60 ± 0.00 |
|           | E310D   | 8.06 ± 0.12 | 1.62 ± 0.10 | 0.01 ± 0.00 | 9.69 ± 0.18 | 4.97 ± 0.28 |
|           | E310Q   | 3.44 ± 0.08 | 0.83 ± 0.01 | 0.02 ± 0.00 | 4.28 ± 0.08 | 4.16 ± 0.14 |

Rates of product formation are reported as nanomoles/nmol of P450/min. Standard deviations are calculated from triplicate measurements.
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FIGURE 7. A, homology model of human P450 4A11 with lauric acid (slate carbons) docked in the active-site cavity (transparent surface). Other protein and atom colors are as described in earlier figures. Arg-96 on strand 2 of sheet β1 can reside in the active site to interact with fatty acid substrates. Coordinates for the P450 4A11 homology model in PDB format are available as supplemental File P450–4A11-homology-model.pdb. 8, amino acid sequence alignment of human 4A11 (residues 81–100) with rabbit, rat, and mouse 4A enzymes. 4A11 Arg-96 (red) is conserved in most 4A sequences with exceptions where an arginine is aligned with His-88 on the adjacent strand of β-sheet 1 which is also oriented into the active site.

used as indicated by the manufacturer). Lysozyme solution (80 mg/ml in water) was added dropwise to a final concentration of 0.2 mg/ml, and then an equal volume of cold water was added dropwise. After stirring at 4 °C for 45 min, the lysates were centrifuged at 8600 × g for 20 min to pellet spheroplasts. The pelleted spheroplasts were frozen in liquid nitrogen and stored at −80 °C until further use. All of the buffers used for purification of the protein contained 0.5 mM PMSF and 20% glycerol. Spheroplasts were suspended in 50 mM potassium phosphate buffer, pH 7.4, containing 1.5 mg/liter DNase I and protease inhibitors (EDTA-free Pierce protease inhibitor tablet). Octane was added into the spheroplast suspension to a final concentration of 615 μM prior sonication. The sonicated bacterial lysate was cleared at 3100 × g for 15 min. The cleared lysate was solubilized with 0.15% Nonidet P-40 (v/v) and 0.15% sodium cholate (w/v) for 1 h at 4 °C followed by centrifugation at 100,000 × g for 60 min. The supernatant was mixed with nickel-nitrilotriacetic acid-agarose at a ratio of 90 nmol of P450/ml of resin and incubated overnight at 4 °C. The nickel-nitrilotriacetic acid-agarose suspension was loaded into a column and washed sequentially with 10 column volumes of 50 mM potassium phosphate buffer, pH 7.4, containing 0.15% sodium cholate and 0.3 mM octane; 10 column volumes of 50 mM potassium phosphate buffer, pH 7.4, containing 0.3 M potassium chloride, 0.1% sodium cholate, and 0.3 mM octane; and 10 column volumes of 500 mM potassium phosphate buffer, pH 7.4, containing 0.06% sodium cholate, 10 mM histidine, and 0.3 mM octane. The enzyme was eluted with 500 mM potassium phosphate buffer, pH 7.4, containing 0.06% sodium cholate, 0.3 mM octane, and 100 mM histidine. Fractions containing the enzyme as judged by absorption spectrum of the heme Soret peak were pooled. Initial fractions exhibiting absorbance ratio of <0.4 for the Soret band relative to protein absorption at 280 nm were not included. The pooled fractions were concentrated 5-fold by ultrafiltration, and diluted 10-fold with 20% glycerol containing 0.5 mM EDTA, 0.06% sodium cholate, and 0.25 mM octane to reduce the phosphate concentration. This material was applied to a column containing HAP-agarose (2.2 × 4 cm). Following a wash with a buffer containing 50 mM potassium phosphate, pH 7.4, 100 mM NaHEPES, pH 7.4, 0.5 mM EDTA, and 0.25 mM octane, the protein was eluted with a buffer containing 300 mM potassium phosphate, pH 7.4, 100 mM NaHEPES, pH 7.4, 0.5 mM EDTA, and 0.25 mM octane. Pooled fractions were concentrated by centrifugal ultrafiltration and then diluted 30-fold with 100 mM NaHEPES, 50 mM potassium acetate, pH 7.4, containing 0.5 mM EDTA and 0.49 mM octane repeatedly to reduce the final potassium phosphate concentration to <1 μM. When prepared in the absence of octane, P450 4B1 exhibits a Soret peak at 417 nm. In the presence of octane, the maximum absorption of the Soret band shifts to 394 nm indicating that the binding of octane produced a high spin ferric protein. The concentration of the enzyme was determined from the intensity of the Soret band of the reduced carbon monoxide complex using visible absorption difference spectroscopy and an extinction coefficient of 91 A²/mM/cm.

Structure Determination—The P450 4B1 octane complex was crystallized by vapor diffusion equilibration using sitting drops in a 24-well Cryochem plate (Hampton Research). The sitting drop was formed by mixing 1 μl of the octane-bound 4B1 protein (450 μM) with 0.2 μl of a solution that was prepared by combining 3:7 (v/v) aqueous ethylene glycol and a 1:1 (v/v) solution of the protein buffer and 1 μl of the precipitant solution containing 0.2 M tri-sodium citrate and 15% polyethylene glycol 3350. The well contained 0.5 ml of the precipitant solution, and the sealed plate was incubated at 24 °C. Prior to freezing crystals for data collection, 2 μl of a solution that was prepared by combining 3:7 (v/v) aqueous ethylene glycol and a 1:1 (v/v) solution of the protein buffer and the precipitant solution was added to the drop. Crystals were harvested and flash frozen in liquid nitrogen prior to shipment to SSRL. X-ray diffraction data collected from a single crystal at 100 K on SSRL line 12-2 were used for structure determination. XDS (52) was used for integration, and Aimless (53) was used for merging and scaling of the reflections to a limiting resolution of 2.70 Å. Initial phasing was obtained by molecular replacement using Phaser as implemented in Phenix (34) with the P450 46A1 structure PDB code 2Q9F as the search model. Phaser identified a single solution (LLG = 127) with one protein molecule in the asymmetric unit in the P 3 2 1 space group. Autobuild generated an initial partial model with R/Refree of 0.36/0.42. Model building and refinement of the atomic coordinates and B-values employed Coot (35) and Phenix, respectively (34). The protein model was completed for native residues 20–501 with the exception of short gaps in the exterior loops connecting helices E with F and G with H. Electron density was evident for octane in the active site. The
Expression and Purification of Rabbit P450 4B1 Glu-310 Mutants—Plasmids for expression of CYP4B1 Glu-310 mutants in E. coli were generated by PCR amplification of FastBac clones used previously to express the corresponding mutants in insect cells (21). The corresponding segment of pCW-4B1 constructs #1 plasmid for expression of the full-length P450 4B1 protein in E. coli (28) was replaced with the mutated sequence from the FastBac clones. The wild-type and mutant proteins were expressed and purified as described previously (28).

Hydrocarbon Metabolism—Full-length wild-type (100 pmol) and mutant proteins were reconstituted with cytochrome P450 reductase (200 pmol), cytochrome b₅ (100 pmol), and dilauryl phosphatidylcholine (20 μg). The reconstituted enzymes were incubated with 1 mM substrate in 0.900 ml of 100 mM KP, buffer, pH 7.4. Following the addition of 0.100 ml of 10 mM NADPH, the reaction was allowed to proceed for 30 min at 37 °C and then terminated by adding 0.100 ml of an aqueous solution of zinc sulfate (15%, w/v). Following the addition of an internal standard (0.010 ml of 1 mM in methanol), the metabolites were extracted with 0.250 ml of ethyl acetate. After centrifugation, 0.08 ml of the organic layer was combined with 0.020 ml of N,O-bistrifluoroacetamide for analysis.

The metabolites were analyzed by gas chromatography and mass spectrometry (Shimadzu GC-17A) equipped with a 30-m × 0.250-mm capillary XTI-5 column (Restek) using helium as the carrier gas with a constant flow rate of 1 ml/min. The injection port temperature was 250 °C. After 1 min, the oven temperature was raised at a rate of 5 °C/min to 115 °C and then rapidly to 280 °C. Metabolites and authentic standards exhibited the following retention times and m/z ratios: 1-heptanol (10.97 min, m/z 173); 2-heptanol (9.20 min, m/z 117); 3-heptanol (9.04 min, m/z 131); 1-octanol (13.62 min, m/z 187); 2-octanol (11.75 min, m/z 117); 3-octanol (11.50 min, m/z 131); 1-nonanol (16.24 min, m/z 201); 2-nonanol (14.38 min, m/z 117); 3-nonanol (14.09 min, m/z 131); 1-decanol (18.82 min, m/z 215); 2-decanol (16.99 min, m/z 117); and 3-decanol (16.67 min, m/z 131). 1-Octanol was used as an internal standard for heptane and decane assays, and 1-nonanol was used for the octane assay, and 1-decanol was used for the nonane assay. Standard deviations were determined from triplicate incubations for each enzyme assayed.

Ligand Binding—Purified full-length CYP4B1 (0.5 μm) was first reconstituted with extruded liposomes of dilauryl phosphatidylcholine (50 μm). CYP4B1 was then placed into two separate cuvettes containing 50 mM potassium phosphate at pH 7.4 in a final volume of 0.9 ml. After allowing the sample and reference cuvettes to reach room temperature, octane was titrated in from 0 to 5 μM. Difference spectra were obtained over the range 350–500 nm using an Aminco DW2 double beam spectrometer. Estimates of the dissociation constant by non-linear regression using SigmaPlot 8.02 and the following function for the enzyme-substrate complex (ΔA(390–422)) versus octane concentration were described by Morrison (54) and are shown in Equation 1,

$$[ES] = \frac{[E]_0 + [S]_0 + K_s}{\sqrt{[E]_0 + [S]_0 + K_s}^2 - 4[E]_0[S]_0}$$

(Eq. 1)

For ligand binding studies with the truncated P450 4B1, which was used for crystallization, the protein prepared in the presence of lauric acid or heptane was dialyzed using sequential rounds of dilution in ligand-free buffer followed by centrifugal ultrafiltration to concentrate the protein. The ligand-free protein exhibited a visible spectrum typical of a low spin ferric P450 with Soret maximum at 417 nm. Octane was dispersed in methanol and added in small volumes to the protein solution as described for the full-length enzyme. Addition of octane shifts the Soret maximum to the high spin ferric form with maximum absorbance at 394 nm. The concentration dependence of the response was analyzed using the quadratic form of the 1-site binding equation (54) by non-linear least squares fitting using SlideWrite with the assumption that the concentration of the bound ligand was proportional to the change in absorbance relative to the maximum change of absorbance (ΔA(388–422)) for the 1:1 complex with protein.

Homology Modeling—MODELLER (55, 56) was used to generate a homology model of human P450 4A11 using the structure of rabbit 4B1 as the template. The alignment of the two structures over residues 20–504 of the 4B1 structure was
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straightforward based on the similarity of the sequences and single deletion of two residues in P450 4A11 corresponding to residues 429 and 430 of P450 4B1 (supplemental Fig. S1). MODELLER generated loops to span the two gaps in the external loops of the 4B1 structure. Visual comparisons of the 4A11 model with the 4B1 structure in COOT indicated that the correspondence of the regular secondary structures flanking the loops overlaid well with the template structure. The planar heme generated by Modeler was replaced with the covalently bound heme in the template to link the heme to the Glu-323 of heme generated by Modeler was replaced with the covalently bound heme in the template to link the heme to Glu-323 of the model, which corresponds to Glu-310 of P450 4B1. The homology model is available as supplemental File P450–4A11-homology-model.pdb. Automated docking studies employed VINA (57), AUTODOCK TOOLS (58) was used to generate PDBQT files from the coordinates for the homology model and ligand and to define the search space. OPEN BABEL (59) was used to generate PDB files for the ligand from the VINA output. The PDBQT files define rotatable bonds and charges. VINA does not use the electrostatic interactions for scoring but does identify hydrogen bond donors based on polar hydrogens and acceptors based on atom type for scoring purposes. To better weight the strong hydrogen bonds between the fatty acid carboxylate and basic amino acids, the scoring weight for hydrogen bonds was doubled using the input parameter “–hydrogen_weight-1.2” as described in the Vina FAQ.

Author Contributions—E. F. J. and M. H. crystallized the protein, collected X-ray diffraction data, determined the structure of CYP4B1 complexed with octane, and drafted the initial manuscript. A. E. R. and B. R. B. generated and characterized the enzymatic properties of CYP4B1 and selected mutants. A. E. R. provided the expression vector for truncated CYP4B1. All authors analyzed the results, contributed to writing the manuscript, and approved the final version of the manuscript.

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References

1. Hsu, M. H., Savas, U., Griffin, K. J., and Johnson, E. F. (2007) Human cytochrome p450 family 4 enzymes: function, genetic variation and regulation. Drug Metab. Rev. 39, 515–538
2. Hardwick, J. P. (2008) Cytochrome P450 ω-hydroxylase (CYP4F) function in fatty acid metabolism and metabolic diseases. Biochim. Pharmacol. 75, 2263–2275
3. Mortensen, P. B., and Gregersen, N. (1981) The biological origin of ketonic dicarboxylic acids: in vivo and in vitro investigations of the ω-oxidation of C6–C16 monocarboxylic acids in unstarved, starved and diabetic rats. Biochim. Biophys. Acta 666, 394–404
4. Kam, W., Kumanan, K., and Landau, B. R. (1978) Contribution of CYP4F1 to the ω-oxidation of fatty acids. J. Lipid Res. 19, 591–600
5. Björkhem, I. (1978) On the quantitative importance of ω-oxidation of fatty acids. J. Lipid Res. 19, 585–590
6. Edson, K. Z., and Rettie, A. E. (2013) CYP4 enzymes as potential drug targets: focus on enzyme multiplicity, inducers and inhibitors, and therapeutical modulation of 20-hydroxyeicosatetraenoic acid (20-HETE) synthase and fatty acid ω-hydroxylase activities. Curr. Top. Med. Chem. 13, 1429–1440
7. Nakano, M., Kelly, E. J., Wiek, C., Vanherpen, H., and Rettie, A. E. (2012) CYF4V2 in Bietti’s crystalline dystrophy: ocular localization, metabolism of ω-3-polysaturated fatty acids, and functional deficit of the p. H331P variant. Mol. Pharmacol. 82, 679–686
8. Lockhart, C. M., Nakano, M., Rettie, A. E., and Kelly, E. J. (2014) Generation and characterization of a murine model of bietti crystalline dystrophy. Invest. Ophthalmol. Vis. Sci. 55, 5572–5581
9. Ohno, Y., Nakamichi, S., Ohkuni, A., Kanayama, N., Naoe, A., Tsujimura, H., Yokose, U., Sugita, K., Ishikawa, I., Akiyama, M., and Kihara, A. (2015) Essential role of the cytochrome P450CYP4F22 in the production of acylceramide, the key lipid for skin permeability barrier formation. Proc. Natl. Acad. Sci. U.S.A. 112, 7707–7712
10. Fan, F., Muroya, Y., and Roman, R. J. (2015) Cytochrome P450 eicosanoids in hypertension and renal disease. Curr. Opin. Nephrol. Hypertens. 24, 37–46
11. Wu, C. C., Gupta, T., Garcia, V., Ding, Y., and Schwartz, M. L. (2014) 20-HETE and blood pressure regulation: clinical implications. Cardiol. Rev. 22, 1–12
12. Laffer, C. L., Gainer, J. V., Waterman, M. R., Capdevilla, J. H., Laniado-Schwartzman, M., Nasjletti, A., Brown, N. J., and Eljovich, F. (2008) Thiazovivoc polymorphism of CYP4A11 and 20-hydroxyeicosatetraenoic acid in essential hypertension. Hypertension 51, 767–773
13. Laffer, C. L., Eljovich, F., Eckert, G. J., Tu, W., Pratt, J. H., and Brown, N. J. (2014) Genetic variation in CYP4A11 and blood pressure response to mineralocorticoid receptor antagonism or ENaC inhibition: an exploratory pilot study in African Americans. J. Am. Soc. Hypertens. 8, 475–480
14. Groves, J. T., and Mcclusky, G. A. (1978) Aliphatic hydroxylations by highly purified liver microsomal cytochrome P-450. Evidence for a carbon radical intermediate. Biochem. Biophys. Res. Commun. 81, 154–160
15. Korzekwa, K. R., Jones, J. P., and Gillette, J. R. (1996) Theoretical studies on cytochrome P-450 mediated hydroxylation: a predictive model for hydroxyl atom abstractions. J. Am. Chem. Soc. 112, 7042–7046
16. de Visser, S. P., Kumar, D., Cohen, S., Shacham, R., and Shaik, S. (2004) A predictive pattern of computed barriers for C-H hydroxylation by compound I of cytochrome p450. J. Am. Chem. Soc. 126, 8362–8363
17. Olsen, L., Rydberg, P., Rod, T. H., and Ryde, U. (2006) Prediction of activation energies for hydrogen abstraction by cytochrome p450. J. Med. Chem. 49, 6489–6499
18. Johnston, J. B., Ouellet, H., Podust, L. M., and Ortiz de Montellano, P. R. (2011) Structural control of cytochrome P450-catalyzed ω-hydroxylation. Arch. Biochem. Biophys. 507, 86–94
19. LeBrun, L. A., Hoch, U., and Ortiz de Montellano, P. R. (2002) Autocatalytic mechanism and consequences of covalent heme attachment in the cytochrome P450A family. J. Biol. Chem. 277, 12755–12761
20. LeBrun, L. A., Xu, F., Kroetz, D. L., and Ortiz de Montellano, P. R. (2002) Covalent attachment of the heme prosthetic group in the CYP4F cytochrome P450 family. Biochemistry 41, 5931–5937
21. Zheng, Y. M., Baer, B. R., Kneller, M. B., Hene, K. R., Kunze, K. L., and Rettie, A. E. (2003) Covalent heme binding to CYP4B1 via Glu310 and a carbocation porphyrin intermediate. Biochemistry 42, 4601–4606
22. Ortiz de Montellano, P. R. (2008) Mechanism and role of covalent heme binding in the CYP4F family of P450 enzymes and the mammalian peroxi-
23. D. Drug. Metab. Rev. 40, 405–426
24. Stark, K., Dostalek, M., and Guengerich, F. P. (2008) Expression and purification of orphan cytochrome P450 4X1 and oxidation of anandamide. FEBS J. 275, 3706–3717
25. Zöllner, A., Dragan, C. A., Pistorius, D., Müller, D., Bode, H. B., Peters, F. T., Maurer, H. H., and Bureik, M. (2009) Human CYP4Z1 catalyzes the in-chain hydroxylation of lauric acid and myristic acid. Biol. Chem. 390, 313–317
26. Stark, K., Wongsud, B., Burman, R., and Oliw, E. H. (2005) Oxygenation of polyunsaturated long chain fatty acids by recombinant CYP4F8 and CYP4F12 and catalytic importance of Tyr-125 and Gly-328 of CYP4F8. Arch. Biochem. Biophys. 441, 174–181
26. Wolf, C. R., Statham, C. N., McMenamin, M. G., Bend, J. R., Boyd, M. R., and Philpot, R. M. (1982) The relationship between the catalytic activities of rabbit pulmonary cytochrome P-450 isozymes and the lung-specific toxicity of tetrafuram derivative, 4-ipomeanol. \textit{Mol. Pharmacol.} \textbf{22}, 738–744

27. Baer, B. R., and Rettie, A. E. (2006) CYP4B1: an enigmatic P450 at the interface between xenobiogenic and endobiotic metabolism. \textit{Drug Metab. Rev.} \textbf{38}, 451–476

28. Cheesman, M. J., Baer, B. R., Zheng, Y. M., Gillam, E. M., and Rettie, A. E. (2003) Rabbit CYP4B1 engineered for high-level expression in \textit{Escherichia coli}: ligand stabilization and processing of the N-terminus and heme prosthetic group. \textit{Arch. Biochem. Biophys.} \textbf{416}, 17–24

29. Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E. L. (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. \textit{J. Mol. Biol.} \textbf{305}, 567–580

30. Jenkins, C. M., and Waterman, M. R. (1994) Flavodoxin and NADPH-flavodoxin reductase from \textit{Escherichia coli} support bovine cytochrome P450c17 hydroxylase activities. \textit{J. Biol. Chem.} \textbf{269}, 27401–27408

31. Gasser, R., and Philpot, R. M. (1989) Primary structures of cytochrome P-450 isozyme 5 from rabbit and rat and regulation of species-dependent expression and induction in lung and liver. Identification of cytochrome P-450 gene subfamily IVB. \textit{Mol. Pharmacol.} \textbf{35}, 617–625

32. Jefcoate, C. R. (1978) Measurement of substrate and inhibitor binding to microsomal cytochrome P-450 by optical-difference spectroscopy. \textit{Methods Enzymol.} \textbf{52}, 258–279

33. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) Phaser crystallographic software. \textit{J. Appl. Crystallogr.} \textbf{40}, 658–674

34. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., et al. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. \textit{Acta Crystallogr. D Biol. Crystallogr.} \textbf{66}, 213–221

35. Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot. \textit{Acta Crystallogr. D Biol. Crystallogr.} \textbf{66}, 486–501

36. Roberts, B. R., Wood, Z. A., Jónsson, T. J., Poole, L. B., and Karplus, P. A. (2005) Oxidized and synchrotron cleaved structures of the disulide redox center in the N-terminal domain of \textit{Salmonella typhimurium} AhpF. \textit{Protein Sci.} \textbf{14}, 2414–2420

37. Sehnal, D., Svobodová Varěková, R., Berka, P., Pravda, K., Navrátilová, V., Banáš, P., Ionescu, C. M., Otyepka, M., and Koča, J. (2013) MOLE-2.0: advanced approach for analysis of biomacromolecular channels. \textit{J. Cheminform.} \textbf{5}, 39

38. Monk, B. C., Tomasiak, T. M., Keniya, M. V., Huschmann, F. U., Tyndall, J. D., O’Connell, J. D., 3rd, Cannon, R. D., McDonald, J. G., Rodriguez, A., Finer-Moore, J. S., and Stroud, R. M. (2014) Architecture of a single membrane-bound state of cytochrome P450 3A4: structure, depth of insertion, and orientation. \textit{J. Am. Chem. Soc.} \textbf{135}, 8542–8551

39. Baer, B. R., Schuman, J. T., Campbell, A. P., Cheesman, M. I., Nakano, M., Mogulevsky, N., Kunze, K. L., and Rettie, A. E. (2005) Sites of covalent attachment of CYP4 enzymes to heme: evidence for microheterogeneity of P450 heme orientation. \textit{Biochemistry} \textbf{44}, 13914–13920

40. Ortiz de Montellano, P. R. (2016) in \textit{Heme Peroxidases} (Raven, E., and Dunford, B., eds) pp. 1–30, The Royal Society of Chemistry

41. Baer, B. R., Kunze, K. L., and Rettie, A. E. (2007) Mechanism of formation of the ester linkage between heme and Glu310 of CYP4B1: 18O protein labeling studies. \textit{Biochemistry} \textbf{46}, 11598–11605

42. Schoch, G. A., Yano, J. K., Sansen, S., Dansette, P. M., Stout, C. D., and Johnson, E. F. (2008) Determinants of cytochrome P450 2CB substrate binding: structures of complexes with montelukast, triptolizone, felodipine, and 9-cis-retinoic acid. \textit{J. Biol. Chem.} \textbf{283}, 17227–17237

43. Palmer, C. N., Richardson, T. H., Griffin, K. J., Hsu, M.-H., Muehroff, A. S., Clark, J. E., and Johnson, E. F. (1993) Characterization of a cDNA encoding a human kidney, cytochrome P-450 4A fatty acid \(\omega\)-hydroxylase and the cognate enzyme expressed in \textit{Escherichia coli}. \textit{Biochim. Biophys. Acta} \textbf{1172}, 161–166

44. Kabsch, W. (2010) XDS. \textit{Acta Crystallogr. D Biol. Crystallogr.} \textbf{66}, 125–132

45. Evans, P. R., and Murshudov, G. N. (2013) How good are my data and what is the resolution? \textit{Acta Crystallogr. D Biol. Crystallogr.} \textbf{69}, 1204–1214

46. Morrison, J. F. (1969) Kinetics of the reversible inhibition of enzyme-catalysed reactions by tight-binding inhibitors. \textit{Biochim. Biophys. Acta} \textbf{185}, 269–286

47. Sali, A., Potterton, L., Yuan, Y., van Vlijmen, H., and Karplus, M. (1995) Evaluation of comparative protein modeling by MODELLER. \textit{Proteins} \textbf{23}, 318–326

48. Sali, A., and Blundell, T. L. (1993) Comparative protein modelling by satisfaction of spatial restraints. \textit{J. Mol. Biol.} \textbf{234}, 779–815

49. Tronrud, E. D., and Olson, A. J. (2010) AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. \textit{J. Comput. Chem.} \textbf{31}, 455–461

50. Morris, G. M., Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S., and Olson, A. J. (2009) AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. \textit{J. Comput. Chem.} \textbf{30}, 2785–2791

51. O’Boyle, N. M., Barcik, M., James, C. A., Morley, C., Vandermeersch, T., and Hutchison, G. R. (2011) Open Babel: An open chemical toolbox. \textit{J. Computinform.} \textbf{3}, 33

52. Gotob, O. (1992) Substrate recognition sites in cytochrome P450 family 2 (CYP2) proteins inferred from comparative analyses of amino acid and coding nucleotide sequences. \textit{J. Biol. Chem.} \textbf{267}, 83–90