Accelerated Publication

The Structure of Human Microsomal Cytochrome P450 3A4 Determined by X-ray Crystallography to 2.05-Å Resolution

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The structure of P450 3A4 was determined by x-ray crystallography to 2.05-Å resolution. P450 3A4 catalyzes the metabolic clearance of a large number of clinically used drugs, and a number of adverse drug-drug interactions reflect the inhibition or induction of the enzyme. P450 3A4 exhibits a relatively large substrate-binding cavity that is consistent with its capacity to oxidize bulky substrates such as cyclosporin, statins, taxanes, and macrolide antibiotics. Family 3A P450s also exhibit unusual kinetic characteristics that suggest simultaneous occupancy by smaller substrates. Although the active site volume is similar to that of P450 2C8 (PDB code: 1PQ2), the shape of the active site cavity differs considerably due to differences in the folding and packing of portions of the protein that form the cavity. Compared with P450 2C8, the active site cavity of 3A4 is much larger near the heme iron. The lower constraints on the motions of small substrates near the site of oxygen activation may diminish the efficiency of substrate oxidation, which may, in turn, be improved by space restrictions imposed by the presence of a second substrate molecule. The structure of P450 3A4 should facilitate a better understanding of the substrate selectivity of the enzyme.

Determination of the structure of P4501 3A4 is of particular interest because the enzyme contributes extensively to human drug metabolism due to its high level of expression in liver (1) and broad capacity to oxidize structurally diverse substrates (2, 3). The enzyme also provides a significant barrier to the bioavailability of new drug candidates contributing to attrition from the developmental pipeline. Additionally, metabolic drug-drug interactions between substrates and inhibitors of the enzyme can profoundly affect the safety or efficacy of drug therapy (4, 5).

Our laboratory was the first to demonstrate that microsomal P450s could be crystallized for structural determination by x-ray crystallography when the proteins were modified for expression as conditional membrane proteins (6, 7). As a result, structures for P450s in family 2, subfamilies B and C are now available (8–14). P450s of family 3, subfamily A exhibit less than 40% amino acid sequence identity with family 2 P450s. In addition, family 3 P450s often exhibit complex kinetic properties such as substrate and effector activation. Effectors or alternative substrates can modulate the apparent binding affinity for other inhibitors (15) and substrates (16). Moreover, there are a number of examples where alternative substrates fail to inhibit the oxidation of specific substrates leading to kinetic models based on the occupancy of the substrate-binding cavity by two substrates that each can be oxidized by the reactive, hypervalent oxy-perferryl heme intermediate without interference from the other (17, 18). The observation that P450 3A4 oxidizes some of the largest substrates identified for P450s, such as cyclosporin, bromocryptine, and macrolide antibiotics (3), has generally suggested the likelihood that the active site cavity of the enzyme is relatively large compared with other P450s. A large active site cavity would also be consistent with models where two or more molecules of smaller substrates are postulated to simultaneously occupy the active site cavity potentially altering the dissociation constant for substrate binding and/or catalytic efficiency by constraining the substrate close to the reactive oxygen intermediate during catalysis. The structure of human microsomal P450 3A4 described in this report exhibits a relatively large substrate-binding cavity that is consistent with these notions regarding...
the enzyme. Of the structures determined previously for family 2 P450s, 3A4 is most similar to that of P450 2C8 (PDB code: 1PQ2) that also oxidizes relatively large substrates and exhibits a large active site cavity. However, the two enzymes differ considerably in the architectures of their active sites in ways that are likely to correspond to the unusual kinetic properties of P450 3A4.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—The pSE3A4His expression plasmid (19) was obtained from James Halpert (University of Texas Medical Branch, Galveston, TX) for the expression of P450 3A4 with a C-terminal His tag. The Bacillus subtilis or Escherichia coli cells were transformed to express P450 3A4 without the trans-membrane leader sequence of the microsomal protein, amino acid residues 3–23, to improve its solubility and facilitate crystallization. No other mutations were employed. The microsomal protein, amino acid residues 3–285, could not be modeled. This is likely to reflect the flexibility of this side of residues 106–217 and 237–242 that connect the F and G helices to helices F’ and G’. The structure is most similar to the structures of mammalian family 2 P450s, which exhibit less than 40% sequence identity with P450 3A4. Eukaryotic P450s, including 3A4, exhibit longer sequences between helices F and G that generally exhibit two additional helices, F’ and G’. This region is thought to form a membrane interaction domain (7), and as seen in family 2 P450s, the outer surfaces of helices F’ and G’ are hydrophobic. 2C8 was chosen for comparison in Fig. 1B because it shares a capacity with 3A4 to oxidize relatively large substrates such as taxanes and statins. The solvent-accessible surfaces of the active site cavities are rendered as a mesh in Fig. 1, A–C. The cavities of 2C8 and 3A4 exhibit similar volumes of 1438 Å³ (8) and 1386 Å³, respectively, when truncated at the narrowest constrictions of the solvent channels. The shapes of the cavities differ in ways that are likely to affect substrate selectivity and enzyme catalysis, and this, in turn, reflects differences in the secondary and tertiary structure of the proteins. The structure of 3A4 is much more open in the vicinity of the heme iron, and the cavity volume is more uniformly distributed than the sinusoid cavity of 2C8. The larger volume of the active site in the vicinity of the heme iron when compared with 2C8 reflects changes in the conformation of the protein in the SRS5 region as it passes from helix K to strand β1–4 as well as structural differences in the SRS2 and SRS3 regions that pass above the heme (Fig. 1, C and D). A large cavity extends outward along the surface of sheet β1 under helix F’ where a relatively large solvent channel to the surface exits below sheet β-1, the end of helix F’ and SRS1 (Fig. 1, A and C). Arg-106 extends across one edge of the channel and participates in a hydrogen-bonding network formed by Arg-106, Glu-374, Asp-76, Arg-372, Asp-61, and Tyr-53 that is stabilized by charge interactions (Fig. 1E). A smaller solvent channel exits the active site cavity on the other side of residues 106–108.

In contrast to the structures of family 2 P450s, helices F’ and G do not pass over the active site cavity in 3A4 (Fig. 1, A and B) because these helices are shorter. Residues 209–217 and 237–242 that connect the F and G helices to helices F’ and G’ form the roof of the active site cavity, are part of SRS2 and SRS3, respectively, and do not exhibit a regular secondary structure (Fig. 1D). A third solvent channel exits the active site cavity under the SRS2 region between SRS4 and SRS6. Several phenylalanine residues that include Phe-108, Phe-213, Phe-215, Phe-241, and Phe-304 contribute to the packing that closes the roof of the active site above the heme between the SRS1, SRS2, SRS3, and SRS4 regions. Interestingly, the reported mutations L211F and D214E located on the outer edge of the SRS2 region above the third solvent channel alter the homotropic cooperativity exhibited by 3A4 catalyzed steroid hydroxylations and confer a hyperbolic dependence on substrate concentration (16). This effect was thought to arise from a reduction of the active site volume due to the increased volume occupied by the side chains of the mutated residues. These residues are not directly in the active site of the structure determined here, and
it is difficult to predict how these mutations would affect protein conformation. Mutations to additional residues in this region, such as Leu-210, Phe-213, and Phe-215, that form the roof of the active site cavity have also been shown to alter the cooperativity exhibited by the enzyme (19, 25). We suspect that the atypical structure of the region forming the roof of the active site cavity could accommodate the simultaneous binding of multiple substrate molecules because of its flexibility.

Although erythromycin was present during crystallization of the protein, the solvent-accessible surfaces of the active site cavities were calculated using a 1.4-Å probe with the program VOIDOO (37) and are rendered as a mesh surface. Electron density maps did not confirm its presence in the crystallized protein.
crystallization buffer, nonspecific binding to micelles of the detergent CHAPS, extraction into the solutions used to protect the crystal during freezing, or extraction of erythromycin into the paraffin oil that was used to reduce the rate of vapor phase equilibration during crystallization. Automated docking studies using the model indicated that the active site cavity was sufficiently large to accommodate the substrate in an orientation that positioned the dimethylamino group of the amino sugar appropriately for oxidation (Fig. 1E) without altering the positions of any amino acids. The figure also displays several amino acid side chains that line the active site cavity and that contact the docked erythromycin molecule. The residues shown in Fig. 1E include residues 119, 301, 304, 305, 369, 370, and 374 that have been implicated as active site residues by site directed mutagenesis studies (26–30).

Another unusual feature of P450 3A4 is the presence of Arg-212 in the active site cavity. The side chain is positioned to directed mutagenesis studies (26–28) using the model indicated that the active site cavity was crystallization buffer, nonspecific binding to micelles of the contact the docked erythromycin molecule. The residues shown amine acid side chains that line the active site cavity and that positions of any amino acids. The figure also displays several amino acid side chains that line the active site cavity and that contact the docked erythromycin molecule. The residues shown in Fig. 1E include residues 119, 301, 304, 305, 369, 370, and 374 that have been implicated as active site residues by site directed mutagenesis studies (26–30).

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