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Deciphering Substrate Recognition by Drug-Metabolizing Cytochromes P450

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It is an honor to be the recipient of the 2002 Bernard B. Brodie Award in Drug Metabolism from the American Society for Pharmacology and Experimental Therapeutics. In receiving this award, I join a distinguished group of honorees (Coon, 1981; Jerina, 1983; Manning, 1986; Nebert, 1988; Levin, 1990; Kalow, 1990; Ziegler, 1991; Gillette, 1991; Guengerich, 1993; Ortiz de Montellano, 1995; Lu, 1998) including Jim Gillette, the 1991 recipient, to whom this issue of Drug Metabolism and Disposition is dedicated. It is particularly fitting that we honor his seminal contributions to the field of drug metabolism on the occasion of the 30th Anniversary of the Journal.

When the first issue of Drug Metabolism and Disposition appeared, P450 research had entered the initial stages of the biochemical and physical characterization of highly purified, individual cytochrome P450 proteins. The true number of drug-metabolizing P450s was unknown, and in sharp contrast to the information available today, the estimated number of drug-metabolizing P450s was small. The nomenclature was also simple: P450 and P448 identified the two distinct xenobiotic-metabolizing enzymes known at the time. The numbers and significance of individual P450s was to unfold first through isolation and characterization of individual enzymes and later through cDNA cloning and heterologous expression. This progress was extensively documented in Drug Metabolism and Disposition. With the completion of whole genomes, we now know that the mouse and human genomes contain 84 and 63 P450 genes, respectively, and that genetic variation on drug metabolism.

Almost 35 years elapsed between the first isolation of a homogenous microsomal P450 and experimental determination of the atomic structure of a microsomal P450, rabbit CYP2C5 (Williams et al., 2000). As the first microsomal P450 to be crystallized, CYP2C5 has provided the first opportunities to characterize structural features that contribute to substrate binding and adaptive changes in enzyme conformation that contribute to recognition of multiple drug substrates that differ in size and properties. This article offers an overview of these studies and their implications for understanding structural determinants of substrate selectivity and metabolism by drug-metabolizing P450s.

CYP2C5 (originally designated form 1) was first isolated as a progesterone 21-hydroxylase that exhibited a genetically determined, elevated expression in liver microsomes prepared from roughly 33% of outbred rabbits (Dieter et al., 1982; Johnson et al., 1989). At that time, efforts in our laboratory and others began to focus on the isolation and identification of hepatic steroid hydroxylases that were not induced by phenobarbital or 2,3,7,8-tetrachlorodibenzo-p-dioxin. The nonresponsiveness to inducers and distinct catalytic activities of the liver steroid hydroxylases provided one of the early clues that as yet unidentified P450s were expressed in liver microsomes. These studies also led to the isolation of CYP2C5 and a related enzyme, CYP2C3 (originally designated form 3(b)), a progesterone 6β- and 16α-hydroxylase, that was also found to exhibit genetic variations in activity (Johnson, 1980; Dieter and Johnson, 1982; Johnson et al., 1989; Hsu et al., 1993). Additionally, CYP2C3-mediated steroid hydroxylation exhibited allosteric activation by structurally related steroids that were not necessarily substrates or inhibitors (Johnson et al., 1983). The genetic polymorphisms exhibited by CYP2C3 and CYP2C5 foreshadowed subsequent characterization of the genetic polymorphism of the human CYP2C enzymes and the impact of this genetic variation on drug metabolism.

Although xenobiotics such as 2-acetylaminofluorene (McManus et al., 1984) and benzo[a]pyrene (Raun and Johnson, 1985) were known to be substrates for CYP2C5, the metabolism of substrates that are oxidized by the human CYP2C enzymes had not been extensively examined. To better understand the relation of the structure of CYP2C5 to its human drug-metabolizing counterparts, CYP2C5 has recently been characterized using several substrates and inhibitors of the human CYP2C enzymes. These studies identified 4-methyl-N-methyl-N-(2-phenyl-2H-pyrazol-3-yl)benzenesulfonamide (DMZ) as a common substrate that is efficiently metabolized by CYP2C5 and the four human CYP2C enzymes (Marques-Soares et al., 2003). DMZ is a nonparallel analag of sulfaphenazole, a relatively selective inhibitor of CYP2C9. The aromatic amino group of sulfaphenazole is replaced with a methyl group in DMZ, and the sulfonamide moiety is methylated. CYP2C5 was also shown to catalyze the 4′-hydroxylation of diclofenac with a catalytic efficiency and regioselectivity that is similar to that of CYP2C9 (Wester et al., 2003b). In contrast, the other human 2C enzymes do not exhibit regioselective oxidation of diclofenac as both of the aromatic rings of diclofenac are hydroxylated with a much lower catalytic efficiency (Marques-Soares et al., 2003). Although the human 2C enzymes oxidize progesterone with a similar regioselectivity for 21-hydroxylation, they exhibit relatively low catalytic efficiencies with only CYP2C19 and CYP2C9 exhibiting sig-
significant rates (Richardson et al., 1995; Yamazaki and Shimada, 1997). Based on these findings, the structures of modified CYP2C5 with DMZ, diclofenac, and progesterone bound in the active site were determined experimentally to 2.3-, 2.1-, and 2.7-Å resolution, respectively. These substrates differ in size, rigidity, shape, and polarity, and the results reveal a role for adaptive changes in enzyme conformation and active site hydration in substrate recognition.

General Features of Microsomal P450 Structure and Adaptations for Membrane Binding

The structure of CYP2C5 indicates that the catalytic domains of microsomal P450s exhibit the conserved folding pattern found for soluble, bacterial P450s even though they exhibit relatively low sequence conservation (<20%) (Williams et al., 2000). The secondary and tertiary structure of CYP2C5 is shown in Fig. 1. Letters and Arabic numerals, respectively, designate helices and sheets (arrows) based on conventions established for bacterial P450s. Figure 1 depicts the familiar “heart”-shaped face of the substrate-binding side of the protein as well as an alternative perspective displaying a side view. The structure exhibits helix (blue)- and β-sheet (tan)-rich regions that flank opposite edges of the heme prosthetic group. The heme and the long internal helix I provide easily recognized visual cues for orientation of the structures shown in subsequent figures.

When compared to the prokaryotic P450s, the structure of CYP2C5 exhibits distinct features for membrane binding and redox partner interactions that are likely to be conserved in other eukaryotic, membrane P450s. The most significant difference between the mammalian P450s and the soluble prokaryotic P450s that is evident in the two perspectives displayed in Fig. 1 is the longer length of the polypeptide chain between helices F and G that includes helices F’ and G’ in CYP2C5. Another difference is an insertion in the “meander region” located between β-sheet 1 and the heme binding site.

Mammalian P450s also exhibit significant N-terminal extensions of the polypeptide chain when compared with soluble prokaryotic P450s. These N-terminal sequences are related to membrane binding and/or organelle targeting. The N-terminal amino acid sequences of representative drug-metabolizing P450s are aligned in Fig. 2. In microsomal P450s, the N-terminal sequence targets the nascent polypeptide for insertion into the endoplasmic reticulum during protein synthesis (Sakaguchi and Omura, 1993). Insertion of the polypeptide is halted at the end of a stretch of roughly 20 hydrophobic amino acid residues that is likely to constitute a single transmembrane helix that extends across the phospholipid bilayer (Vergéres et al., 1989). Antibody epitope mapping studies indicate that the catalytic domain is largely exposed on the cytoplasmic surface of the endoplasmic reticulum (reviewed in Von Wachenfeldt and Johnson, 1995) and, thus, is not likely to be closely associated with the transmembrane helix that spans the membrane, as depicted in Fig. 3. A linker region that is rich in basic amino acid residues connects the transmembrane helix to a proline-rich motif found at the beginning of the catalytic domain (Fig. 2). Monoclonal antibodies that specifically recognize the linker region of CYP2B4 indicate that this region is exposed on the cytoplasmic surface of the endoplasmic reticulum.
The N-terminal transmembrane helix and the linker region (forest green) were not present in the structure determined for the DMZ complex of CYP2C5 (PDB: 1N6B). The transmembrane helix was modeled to span the membrane. The conformation of the linker region is unknown and is represented in an extended conformation for clarity. The hydrophobic tip of the catalytic domain formed by the helix F′ to G′ region and the region before helix A are likely to interact with the membrane as discussed in the text. The protein is rendered as described in the legend to Fig. 1. The phospholipid bilayer is rendered with CPK-colored atoms with carbons colored white. The figure was rendered using MOLSCRIPT (Kraulis, 1991) and RASTER3D (Merritt and Bacon, 1997).

The catalytic domains of mammalian P450s are also likely to interact directly with membranes. In the case of mitochondrial P450s, the N-terminal sequences are removed upon import into mitochondria where these enzymes are bound to the inner membrane (Omura and Ito, 1991). Alignments of the amino acid sequences of mitochondrial P450s with those of microsomal P450s do not reveal a distinctive insertion of an apparent transmembrane sequence within the catalytic domain. Detergents are required to disassociate mitochondrial P450s from the inner membrane, indicating a likelihood that their catalytic domains interact extensively with the hydrophobic core of the membrane bilayer.

Truncated microsomal P450s such as those crystallized in our laboratory also bind peripherally to phospholipid membranes (Cosme and Johnson, 2000). However, high salt buffers can disrupt this binding to membranes, indicating that the interaction of the catalytic domain with the membrane is less extensive than that of mitochondrial P450s. Estimations of the height of microsomal CYP2B4 above the membrane surface by atomic force microscopy (Bayburt and Sligar, 2002) as well as the area of phospholipid monolayers displaced by the binding of CYP2B4 (Shank-Retzlaff et al., 1998) indicate that a portion of the catalytic domain of microsomal P450s is likely to be buried in the membrane. The hydrophobicity of the catalytic domain near its site of attachment to the N-terminal linker region suggests that this portion of the catalytic domain is the most likely to be buried in the phospholipid membrane. This hydrophobic surface is formed by the juxtaposition of the regions between the proline-rich motif and helix A as well as regions between helices F and G (Williams et al., 2000). Epitope-specific antibody binding to the latter region is blocked or diminished when the protein is bound to the membrane (De Lemos-Chiarandini et al., 1987), indicating that the region between helices F and G resides in or near the membrane surface. In addition, mutations in the helix F and F′ region diminish the binding of truncated CYP2C5 to membranes (Cosme and Johnson, 2000). These mutations along with the N-terminal truncations also facilitated the initial crystallization of CYP2C5. This protein is designated 2C5/3LVdH. Figure 3 illustrates a likely orientation of the catalytic domain relative to a phospholipid bilayer that reflects the information discussed. In addition, the tilt of the heme corresponds to estimates for microsomal P450s CYP17A and CYP21B, based on the rate of decay of the absorption anisotropy following photodissociation of carbon monoxide complexes of each protein (Ohta et al., 1992).

**Electron Transfer Interface**

Mutagenesis experiments indicate that redox partners interact with the proximal surface of P450s. The interactions with the membrane that anchor the catalytic domain in a tilted orientation are likely to increase the likelihood of productive encounters between P450 reductase and the proximal face of the P450 as the two proteins diffuse along the membrane surface. The structure of the proximal face of the catalytic domain is more highly conserved than the distal surface and is largely formed by the structural core of the enzyme that provides the heme binding site. This conserved architecture maintains a high similarity for the reductase binding site among functionally divergent P450s.

The structure of the proximal surface of CYP2C5 is most similar to that of the soluble, prokaryotic enzyme CYP102 (Williams et al., 2000). In contrast to most soluble, prokaryotic P450s, which utilize ferredoxin-like iron sulfur proteins as electron donors, CYP102 is fused to an FMN- and FAD-containing flavoprotein domain that resembles microsomal P450 reductase. CYP102 exhibits an insertion between the end of β-sheet 1 and helix L that is not generally seen in prokaryotic P450s that utilize iron sulfur proteins as electron donors. This insertion was termed the meander region (Hasemann et al., 1995), and this region is generally longer in microsomal P450s.

The proximal surface of CYP2C5 is relatively electrostatically positive when compared with the distal surface (Williams et al., 2000), and the positive electrostatic potential of the proximal surface appears to contribute to reductase binding. A number of positively charged residues on the proximal face of CYP2C5 are highly conserved in family 2 P450s. Individual mutations to the corresponding residues in CYP2B4 diminishes the apparent affinity of the P450 for reconstituted reductase (Bridges et al., 1998). The positions of these critical residues are shown in Fig. 4.

The structure of CYP102 complexed with the FMN domain of the flavoprotein moiety of the enzyme (Sverioukova et al., 1999b) suggests an interesting model for the interaction of microsomal P450s with the microsomal P450 reductase (Poulos and Johnson, 2004). In this structure, the FMN domain of the reductase binds to the proximal surface of CYP102 so that the FMN is close to the heme. The binding is anchored on one side by interactions of the FMN domain with helix C and on the other side of the heme by the meander region. The structure is consistent with mutagenesis data and the proposed role of electrostatic forces in the interaction (Sverioukova et al., 1999a).
places the FMN in close proximity to the Fe for electron transfer. However, the structure of the microsomal P450 reductase (Wang et al., 1997) reveals a conformation in which the FMN domain interacts with the FAD domain of the reductase that would exclude the proposed interaction with P450s. The FAD and FMN domains are connected by a long, flexible linker, leading to suggestions that the FMN domain may swing away from the FAD domain to interact with microsomal P450s (Poulos and Johnson, 2004).

Heme Binding Site

The heme prosthetic group represents the catalytic center of P450 enzymes because the heme transfers an oxygen atom from a donor to the substrate. In microsomal drug-metabolizing enzymes, molecular oxygen is generally the source of the oxygen atom, and the reduction, protonation, and scission of molecular oxygen occurs while it is bound to the heme iron. One edge of the heme is sandwiched between helix I on the substrate-binding side and a β-helix preceding helix L on the opposite side (Fig. 5). The thiolate side chain of a conserved cysteine residue, C432 in CYP2C5, found in the turn preceding helix L is the axial ligand for the heme iron, as illustrated for CYP2C5 in Fig. 5. Thr298, a highly conserved residue, participates in the hydrogen-bonding interactions that distort helix I in many P450s (Poulos and Johnson, 2004), including CYP2C5. The structure of the peroxo anion complexed to the heme Fe of CYP101 (Schlichting et al., 2000) indicates that the oxygen anion intermediate is oriented toward the cleft. The bound waters and a highly conserved acidic residue adjacent to Thr298 are thought to provide a proton transfer chain to the oxygen dianion and hydroperoxy anion intermediates (Gerber and Sligar, 1994). Changes in the positions of these waters and the side chain of the acidic residue are evident in the structures of the reaction intermediates in CYP101 (Schlichting et al., 2000).

The bound peroxo or hydroperoxy anion intermediates may also provide alternative reactants in some P450-catalyzed reactions such as the final step in the conversion of androgens to form estrogens (Akhtar et al., 1993). Evidence for similar reaction pathways has also been presented for a drug-metabolizing P450, 2B4, and these are favored in mutants of CYP2B4 where the conserved threonine is mutated to an alanine (Coon et al., 1998). This mutation is thought to decrease the rate of proton transfer and thereby increase the lifetime of the peroxo-iron intermediates.

Substrate-Binding Site

Substrates bind on the distal side of the heme in close proximity to the site of oxygen binding (Fig. 5). Substrates are positioned in the active site by several portions of the polypeptide chain that form the substrate-binding cavity. Gotth (1992) described the different portions of the polypeptide chain of CYP101 that form the active site as substrate recognition sites (SRSs), which are numbered 1 through 6 from the N-terminus. These designations have been widely adopted to describe the different portions of mammalian P450 polypeptide chains that are likely to define the substrate-binding cavities of microsomal P450s based on sequence alignments and are consistent with the structure of CYP2C5 (Williams et al., 2000). The portion of helix I
that borders the substrate-binding cavity is designated SRS 4. SRS 5 is directly across the heme surface from SRS 4 and is formed by the loop between helix K and β-sheet 1. These two SRS regions converge to close one end of the substrate-binding cavity on the opposite side of the heme Fe from the bound substrate. SRS 6 is formed by the turn in β-sheet 4 and is positioned above SRS 5. Portions of the N-terminal β-sheet system do not form the substrate-binding site of CYP101 and did not receive an SRS designation. However, the N-terminal β-sheet system does form a portion of the substrate-binding sites of some P450s including CYP102 (Li and Poulos, 1997; Haines et al., 2001) and CYP2C8 (unpublished observation).

The remaining SRS regions are found on the most mobile and varied portions of P450 structures. The SRS 1 region, which corresponds to the portion of the polypeptide chain between helices B and C, passes above the heme propionates and closes the end of the trough/cavity formed by SRS 4, SRS 5, and SRS 6. The conformation of the polypeptide chain in this region, as well as the presence, length, and orientation of the B’ helix, varies greatly among P450s. SRS 2 and SRS 3 are located on helices F and G, respectively. Helices F and G are cantilevered over the active site above the heme with helix I serving as the fulcrum.

Helices B’, F, and G are often positioned differently in various P450s. The diversity of these regions is illustrated in Fig. 6 by the structures of CYP102 from Bacillus megaterium (Ravichandran et al., 1993), CYP154C1 from Streptomyces coelicolor A3(2) (Podust et al., 2003), and CYP51 from Mycobacterium tuberculosis (Podust et al., 2001). In contrast to the structures of CYP2C5, the active sites of the three structures shown are open to bulk solvent, suggesting possible pathways for substrate access. A relatively small opening that leads to the heme in the structure of substrate-free CYP102 is evident between the helix F-G region and β-sheet 1. This opening is sufficiently large for the binding of the hydrophobic end of its fatty acid substrates. The presence of the substrate and large movements of the F and G helices close the structure when substrates bind (Li and Poulos, 1997; Haines et al., 2001). CYP154C1 exhibits a much larger, open cleft (Podust et al., 2003). The helix F-G region of CYP154C1 is pivoted up and away from the heme in the structure of CYP154C1 (Fig. 6). The identity of the natural substrate of CYP154C1 is unknown, but this P450 has been shown to oxidize relatively large macrolide compounds. Helix B’ resides on opposite sides of the substrate access channel in the structures of CYP102 and CYP154C1, so that in CYP154C1, the substrate can enter in a direction that parallels helix I and that is almost perpendicular to that seen for fatty acids in CYP102. A third variation on this theme is seen in the structure of CYP51, where the SRS 1 region is in an extended conformation that is associated with an acute bend of the N-terminal end of helix I. This creates an opening to the active site above the heme propionates, suggesting that substrates could also enter under helix B’. Closed structures such as those determined for CYP2C5 will need to open for substrate access and product egress. The open structures shown in Fig. 6 suggest probable conformations of the helix F-G and helix B’ regions that may provide substrate access.

The complex of CYP2C5 and progesterone (unpublished results) was formed both by the cocrystallization of progesterone with the enzyme and by soaking crystals of the substrate-free enzyme with progesterone. Progesterone is positioned in the same location in each of the complexes within experimental error, < 0.4 Å root mean square. Because large portions of the protein are immobilized by
contacts with other molecules in the crystal, particularly helices F-G and the N-terminal β-sheet system, the protein is most likely to open for substrate binding by adopting a conformation of the helix B to helix C region, similar to that of CYP154C1, because this region is very flexible. As will be discussed later, this region also adapts its conformation to different substrates when they bind.

**Substrate Contact Residues**

Only a few of the amino acid side chains in SRSs actually reside inside the active site cavity and potentially contact substrates. Table 1 lists amino acid side chains that contact progesterone, DMZ, or diclofenac in the experimentally determined structures of each complex with CYP2C5. A relatively generous cutoff distance of 5 Å has been used to identify these residues. This choice is consistent with the formation of contacts at distances that reflect the sum of the van der Waals radii (generally 3–4 Å) as well as residues that are sufficiently close to exclude water molecules from the interface between the residue and the substrate. Although the residues that contact each substrate are often the same, differences are apparent that reflect not only the binding of each substrate in overlapping but distinct locations within the substrate-binding cavity, but also the sizes and shapes of the substrates as well as conformational changes adopted by the enzyme when substrates bind.

A single molecule of DMZ binds to CYP2C5 in two alternate binding conformations and locations, termed AC1 and AC2, that reflect anti-parallel orientations of the long axis of the DMZ molecule (Fig. 7) (Wester et al., 2003a). Although the two locations overlap, the substrate occupies distinct portions of the active site cavity in each orientation, and the cavity is larger than the volume occupied by an individual substrate molecule. As a result, there are differences in the identities of the amino acid side chains that contact DMZ in each binding location and also in the substrate atoms that are contacted by CYP2C5. The AC1 conformation is the most productive for oxidation. Binding of DMZ in the AC1 location orient the benzylic methyl group optimally for hydroxylation of the methyl group (Fig. 7), and this reaction accounts for >98% of the products formed. The alternative AC2 conformation positions DMZ so that the phenyl ring on the other end of the DMZ molecule is closest to the heme iron. However, the phenyl ring is not positioned as close to the heme iron (5.9 Å) in the AC2 orientation as the benzylic methyl group is positioned in the AC1 orientation. Thus, hydroxylation of the phenyl ring yields only a minor fraction (<1%) of the products (Marques-Soares et al., 2003). In contrast, the benzylic methyl group of DMZ is positioned 4.4 Å from the heme Fe (Fig. 7). This optimally places a carbon-hydrogen bond of the methyl group for hydrogen abstraction by the oxygen atom of the oxoperferrylporphyrin cation radical intermediate during catalysis. Substrates are positioned similarly for aliphatic hydroxylation in substrate complexes of the prokaryotic CYP101 (Poulos et al., 1987) and CYP108 (Cupp-Vickery and Poulos, 1995).

In contrast, the 4′-hydroxylation of diclofenac is likely to involve direct addition of the heme-bound oxygen atom to the π-electron system of the dichlorophenyl, aromatic ring. Appropriately, the face of the dichlorophenyl ring of diclofenac is positioned in CYP2C5 to orient the π-electron system toward the site of oxygen binding (Wester et al., 2003b). The 3′ and 4′ carbons are roughly equidistant from the heme Fe at 4.4 and 4.7 Å, respectively (Figs. 5 and 8). After addition of the oxygen atom, the intermediate epoxide would rearrange by generally accepted mechanisms for aromatic epoxidation to form the 4′-hydroxy product (Daly et al., 1972; Guengerich, 2003).

The multiple orientations for substrate binding, such as those exhibited by DMZ bound to CYP2C5, are likely to underlie the obser-

### Table 1

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XXX, contact residue; X, generous contact, 4.5 to 5.0 Å.
vation that other substrates of P450 enzymes are oxidized at more than one site on the substrate by the same P450. In the case of DMZ, which is oxidized on the two distal ends of the molecule, steric constraints will not allow an end-for-end rotation in the active site after DMZ binds to the enzyme. Once DMZ binds in the less productive orientation, it effectively inhibits the binding of DMZ in the more productive orientation for hydroxylation of the benzylc methyl group and reduces the overall catalytic efficiency of the enzyme.

Almost all of the amino acid side chains in the active site of CYP2C5 that contact each of the three substrates are hydrophobic. Hydrophobic interactions are likely to contribute significantly to the free energy of substrate binding in two ways. First, the free energy change resulting from desolvation of the hydrophobic surfaces of the substrate and of the binding site cavity of the enzyme will provide a significant contribution to a favorable free energy change for binding (the hydrophobic effect). Second, the van der Waals interactions that are formed between the substrate and protein provide additional contributions to the stability of the complex. These contributions will be diminished by any loss of hydration that may occur for polar moieties of the substrate or active site amino acids. Unfavorable entropy changes also arise from reductions in rotational degrees of freedom resulting from steric interactions. The side chain interactions with the ketone oxygens of progesterone or the amide nitrogen and sulfonyl oxygens of DMZ are largely hydrophobic.

In contrast, the highly polar carboxyl moiety of diclofenac is hydrated in the structure of the CYP2C5 complex (Fig. 8). Diclofenac is smaller than either DMZ or progesterone, and additional water molecules reside in parts of the binding cavity that are not occupied by diclofenac. The additional waters in the distal portion of the active site form a cluster with extensive hydrogen bonds. These waters also provide hydrogen bonds to the substrate and to polar side chains in the active site cavity. N204, S289, D290, and K241, that contribute to the stability of the complex. Of these amino acid side chains, only D290 remains hydrated in the complex with DMZ because this larger substrate displaces most of the water from the distal portion of the active site. The hydrogen-bonding interactions between the waters, CYP2C5, and diclofenac are likely to contribute to both a favorable binding affinity and a single binding orientation that leads to a high regiospecificity for metabolism.

The carboxyl moiety of diclofenac binds to CYP2C5 in close proximity to the carboxyl side chain of D290. As the pK_a for these carboxylates is <5.0 in solution, the close proximity of the two potential anions is unexpected. The two intervening water molecules provide some dielectric shielding. In addition, one or both of the carboxylates may be protonated in the CYP2C5 complex. Lowering the pH from 7.6 to 5.3 increases the binding affinity 4-fold to 12 μM (Wester et al., 2003b). This value is similar to the K_a exhibited by CYP2C5 for the neutral amide of diclofenac, which is not affected by the difference in pH.

Adaptive Conformation Changes for Substrate Binding

Diclofenac is smaller and more polar than DMZ, and it occupies a smaller portion of the substrate-binding site than does DMZ. As a result, fewer side chains contact diclofenac when it is bound in the active site (Table 1). A comparison of the positions of these side chains reveals several differences that reflect changes in side chain orientation as well as shifts in the peptide backbone of the enzyme (Fig. 9). For example, large changes in the position of the P473 side chain reflect alternative orientations of the side chain and relatively subtle differences in the polypeptide backbone. In contrast, the differences in the location of helix B' produce a 2.5-Å difference in the location of Leu 103. The largest changes in the conformation of the polypeptide backbone are evident for the B' helix and the regions between helices E and F (Wester et al., 2003b). Progesterone is relatively similar in size to DMZ, but it is much more rigid and planar. The location of the B' helix in the progesterone complex with CYP2C5 is more similar to that seen in the DMZ complex than in the diclofenac complex.

The adaptive positioning of helix B', when substrates bind, reflects the relatively weak interactions of this helix with the rest of the protein structure. Helix B' exhibits only one Van der Waals contact with each of the adjacent helices, G and F'. The remaining contacts are with the substrate. Helix B' is also flanked by two Gly X Gly motifs. Because the glycine residues do not have side chains, they are relatively free to adopt a variety of conformations that contribute to the flexibility of this region. The portions of the loops preceding and following the GXG motifs are stabilized by interactions with adjacent portions of the structure. In addition, R97 in the N-terminal GXG motif interacts with the heme propionates. Unpublished work indicates that residue 108 in the middle of the C-terminal GXG motif can adopt different conformations that are dependent on the surrounding
residues. The flexibility of the helix B to C region is also likely to facilitate substrate access from the solvent as discussed earlier.

**Homology Modeling**

Many of the residues listed in Table 1 align with residues in other CYP2 enzymes that have been implicated by mutagenesis studies to be determinants of substrate selectivity, as reviewed previously (Williams et al., 2000; Domanski and Halpert, 2001). In addition, the results of targeted mutagenesis of residues in other drug-metabolizing P450s based on alignments with the sequence of CYP2C5 have generally indicated that these substitutions can alter catalytic activity (Domanski and Halpert, 2001) and, in some cases, transfer the substrate selectivity or regiospecificity of metabolism of one enzyme to another (Kumar et al., 2003).

Although these results facilitate structural alignments of various sequences with that of enzymes with experimentally determined structures, a number of considerations suggest that it will be difficult to build accurate homology models. This is exemplified by the differences in the conformation of the helix B to helix C region as discussed earlier. Additionally, the structures of CYP 2C5, 2C8, and 2C9 (unpublished) exhibit differences both in the lengths of helices and in their positions. These differences are likely to be difficult to predict, a priori. However, the crystallization of additional drug-metabolizing P450s and determination of their structures will increase the accuracy of models for the prediction of substrate selectivity and provide a framework for the analysis of genetic polymorphisms that alter catalytic properties.

**Summary**

The structure of CYP2C5 indicates that the basic fold exhibited by prokaryotic P450s is largely conserved in the structures of microsomal P450s. Organelle targeting is largely achieved by the N-terminal extension of the polypeptide chain seen in eukaryotic P450s. Although the N-terminal transmembrane domain of microsomal P450s provides a membrane anchor, adaptations of the catalytic domain are required to explain the binding of N-terminally truncated microsomal P450s and mitochondrial P450s to membranes. This is likely to reflect significant insertions of hydrophobic residues between helices F and G and the hydrophobicity of the surrounding surface of the protein. The active site of CYP2C5 is largely hydrophobic and can accommodate substrates of varying sizes and shapes. The peptide backbone of regions forming the distal portion of substrate binding cavity is flexible, contributing to adaptations to substrate binding and the opening and closing of substrate and solvent access channels. Although unoccupied space is generally seen when substrates bind, the substrate is largely constrained from executing large-scale rotations within the cavity. Oxidations of alternative sites on the substrate are therefore likely to arise from multiple binding orientations and locations rather than dynamic motion in the active site. Specific polar interactions can contribute to regioselectivity by giving rise to preferred orientations for binding. In addition, residual hydration in the distal portion of the active site cavity can stabilize specific binding orientations. These attributes contribute to the recognition of diverse substrates by drug- and xenobioc-t metabolizing P450s.

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**References**


