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Review

Cytochrome P450: Molecular Architecture, Mechanism, and Prospects for Rational Inhibitor Design

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Cytochromes P450 catalyze the insertion of one O_2-derived oxygen atom into an aliphatic or aromatic molecule. P450s are best known for the metabolism of xenobiotic molecules, where hydroxylation renders insoluble hydrocarbons more soluble for easier elimination. In addition to this important catabolic function, P450s catalyze key steps in steroid and plant growth regulator metabolism. A variety of therapeutic, fungicidal, and agochemical agents that perturb these metabolic pathways very likely operate by binding in the lipophilic P450 active site and coordinating with the heme iron atom. Recent determination of a bacterial P450 crystal structure, P450cam from *Pseudomonas putida*, in addition to the crystal structure of four inhibited complexes, has provided some insight into the potential use of P450 as a model system for the rational design of therapeutic agents. The crystal structure has also shed light on the P450 catalytic mechanism. P450cam operates differently from peroxidase or catalase in cleaving the O–O bond, since unlike these other enzymes, P450 contains no acid–base catalytic groups near the oxygen binding site. Instead, the O_2 pocket is lined with aliphatic and aromatic residues. This strongly suggests that the catalytic push required to cleave the O–O bond resides with the ability of the Cys heme ligand to donate electron density to the heme–oxy system. A comparison of the substrate-free and -bound P450cam crystal structures has revealed some interesting aspects regarding the dynamics of substrate binding. The structures of both forms of P450cam are the same except that in the substrate-free enzyme, the active-site pocket fills with a network of water molecules, one of which coordinates with the iron atom. Despite this lack of any significant conformational rearrangement of protein groups, a careful analysis of crystallographic temperature factors shows dynamical differences. Segments of the protein that are separated in the sequence but that lie close to one another in the structure and that define a small entrance to the substrate pocket undergo significantly higher thermal motion in the substrate-free enzyme. This suggests that dynamical fluctuations at the molecular surface play an important role in controlling substrate binding.

KEY WORDS: cytochrome P450; crystal structure; inhibitor design; catalytic mechanism.

INTRODUCTION

Specific and nonspecific hydroxylation reactions play an essential role in a number of metabolic and catabolic processes. Cytochrome P450, an enzyme of central importance in such reactions, catalyzes the pyridine nucleotide-dependent insertion of one O_2-derived oxygen atom into an aliphatic or aromatic substrate. Cytochrome is perhaps a misnomer since this implies only an electron transfer function when, unlike cytochromes and globins, P450s catalyze bond making and breaking reactions. At the time of its discovery, P450 was recognized as an unusual pigment and not as an enzyme (1,2). The discovery of P450 is itself an interesting story. Initially it was found that when liver microsomes were reduced and purged with carbon monoxide, a strong absorption band at 450 nm resulted, which was most unusual for the then-known pigments (1,2). Thus the name P (for pigment) 450. What seems remarkable, especially considering that P450 is the major heme protein of liver microsomes, comprising about 1.0 nmol/mg microsomal protein (3), is that P450 was not discovered until the late 1950s and was not established as a hemeprotein until 1964 (4).

Since its discovery, research on P450 has undergone an explosive growth, due primarily to three key observations. First, Estabrook and co-workers established the role of P450 in the metabolism of steroids (5); second, Remmer and Merker found that P450 was induced in animals pretreated with various drugs (6); and third, Mason and co-workers discovered a unique electron paramagnetic resonance signal in liver microsomes which (7) later was found to be due to P450. Thus, P450 attracted the interest of both biological and biophysical disciplines including many forms of spectroscopy, enzymology, pharmacology, toxicology, and molecular biology. Perhaps the single property of P450s that ties these disciplines together is the inducibility of specific P450s by different pharmacological agents. The classic example is the phenobarbital and 3-methylcholanthrene induction of microsomal P450s (8), with more recent advances centered on the molecular biology of the murine *Ah* locus involving induction of P450s by aromatic hydrocarbons such

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as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (9). The importance of P450 induction to pharmacologists and molecular biologists is obvious but P450 also is of interest to the enzymologist and biophysical chemist. The unusual spectroscopic properties of P450 and the changes in these properties that occur upon substrate or inhibitor binding have provided fruitful terrain for many spectroscopic probes. The nature of how the enzyme cleaves the O–O bond and then inserts an oxygen atom into an unactivated C–H bond has been a particularly challenging problem. Perhaps most intriguing is the large number of structurally unrelated organic molecules hydroxylated by various P450s despite the fact that the basic redox, spectral, and O–O bond cleavage properties are similar in all P450s.

One additional area of considerable interest is the use of P450 inhibitors as therapeutic agents. The structure of several such agents is shown in Fig. 1. The most well-known agent is metyrapone, used to treat hypercortisolism (Cushing's syndrome; 10). Metyrapone operates by binding at the active site of mitochondrial P450s involved in steroid metabolism, thereby lowering the production of cortisol. A variety of triazole and imidazole compounds blocks ergosterol biosynthesis in fungi, most likely as P450 inhibitors, and are used as medical and agricultural fungicides (11). As with any set of drugs, insecticides, or herbicides, however, the key problem is specificity. The goal is to kill the invading organism but leave the host unaffected. Toward this end, there is growing optimism in the area of rational drug design. If one possesses detailed three-dimensional structural information about the receptor macromolecule, hopefully of both the invader and the host, one is in a position to use computer molecular modeling techniques to design specific inhibitors. P450 offers some promise in this area. After reviewing the structural and catalytic properties of P450 we return to this question of the prospects for the rational design of P450 inhibitors.

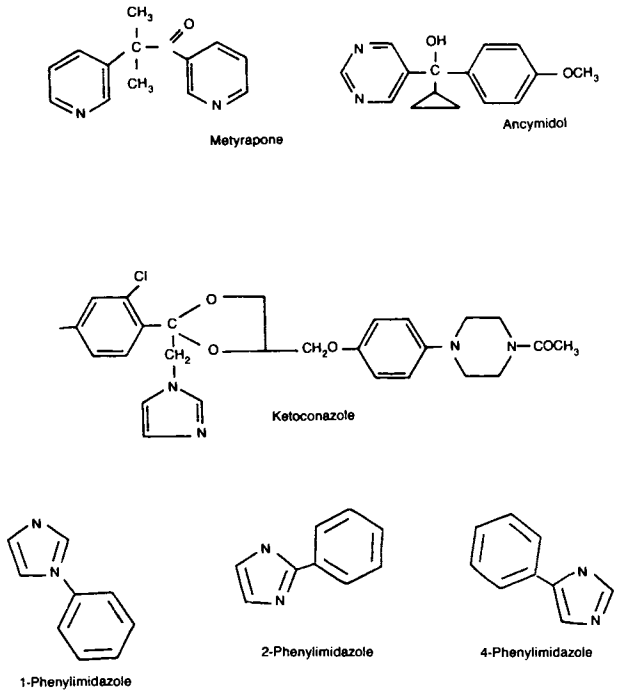

Fig. 1. The structure of various known P450 inhibitors. As noted in the text, metyrapone is used to control conditions where cortisol is hypersecreted. Ketocanazole is a well-known antimycotic drug that blocks P450-dependent ergosterol biosynthesis. The 1, 2, and 4 isomers of phenylimidazole are well-characterized P450cam inhibitors.

MOLECULAR PROPERTIES OF P450

P450s consist of a single polypeptide chain on the order of 45,000 to 55,000 daltons and contain a single, noncovalently bound heme. Most P450s exhibit an absorption maximum near 418 nm, which shifts to near 390 nm when substrate binds. This represents a low-to-high spin transition where, in the substrate-free form, the heme iron atom has all 5 d electrons maximally spin paired to give a net spin of 1/2, while when substrate binds the spin shifts to 5/2. This shift is a classic trademark of P450–substrate interactions and typically is used to follow substrate binding.

Owing to recombinant DNA technology, the number of available P450 sequences continually grows. All P450s thus far sequenced (over 60) exhibit strong homology even when the only complete bacterial P450 sequence is compared with its eukaryotic counterparts. The most striking homology occurs around the heme ligation environment. Table I shows the sequence around one such active-site peptide. P450cam represents the camphor monoxygenase P450 from *Pseudomonas putida,* which currently represents the only complete bacterial sequence (12) and the only known crystal structure of a P450 (13). Cys357 of P450cam operates as one of the axial heme ligands (Fig. 2) and the strong sequence homology around Cys357 provides compelling evidence that the corresponding Cys in all P450s provides one of the heme ligands. The degree of homology extends to the microenvironment surrounding the Cys ligand. For example, Phe350 thusfar is invariant, while a branched aliphatic side chain always occurs at position 359 (P450cam numbering). An invariant Gly (Gly353) occurs at a strategic location for reversing the direction of the polypeptide chain just beneath the heme. It therefore appears that not only is the homologous Cys used as a ligand in all P450s but that the three-dimensional structure surrounding the Cys ligand is conserved in all P450s. The reason for such strict conservation very likely relates to the critical role that the Cys–Fe bond plays in the catalytic mechanism which we consider further on.

Overall Molecular Architecture

Figure 3 shows the overall topography of P450cam derived from the highly refined 1.63-Å X-ray structure (13). Helices account for about half the residues and are divided among 13 different helical segments. The beta structure occurs mainly as isolated antiparallel beta pairs with no extended sheet structure. Thus, P450 is similar to the globins and cytochrome *c* peroxidase, where helices dominate the overall fold. Notice that P450cam divides into two domains, with most of the helices clustered together on the right half of the molecule while most of the beta structure occurs on the left half.

The heme is completely sequestered, with only the carboxylates of the heme propionates solvated. Helix I lies directly over the heme and, together with helix C and one strand of beta 3, provides hydrophobic heme contacts. As

Table I. Alignment of Sequences in the Vicinity of the Thiolate Ligand

P450cam	F	G	H	G	S	H	L	C_{357}L	G	Q	H	L	A	R
P450C (rat)	F	G	L	G	K	R	R	C_{461}I	G	E	T	I	R	R
P450LM2 (rabbit)	F	S	L	G	K	R	I	C_{436}L	G	E	G	I	A	R
P450b,e (rat)	F	S	T	G	K	R	I	C_{436}L	G	E	G	I	A	R
P450scc (bovine)	F	G	W	G	V	R	Q	C_{466}V	G	R	R	I	A	E

noted above, the sulfur atom of Cys357 coordinates with the iron atom, while in the substrate-bound, high-spin state, the remaining axial coordination position *trans* to the Cys ligand remains vacant (Fig. 2). The crystal structure work (14) has shown that when the substrate is removed, the low-spin iron atom becomes hexacoordinate with a water molecule or hydroxide ion *trans* to the Cys ligand (Fig. 4). Moreover, water molecules enter the active site to occupy the space formally taken by the substrate, camphor. There are no large structural changes that accompany substrate binding, although there are dynamical differences. A detailed analysis of crystallographic temperature or Debye–Waller factors shows that three regions of the enzyme undergo greater dynamical fluctuations in the absence of substrate (14). Although these regions are well separated in the sequence, they are positioned close to one another in the three-dimensional structure and they define a small opening that connects the molecular surface with the active site. Thus, we have postulated that dynamical fluctuations at the molecular surface play an important role in substrate binding.

A question of central importance is How closely does the overall structure of eukaryotic P450s match the P450cam structure? The only clear-cut structural information we have relevant to this question is one crystal structure (P450cam) and a large number of eukaryotic P450 sequences. A comparison between these sequences demonstrates a relationship between P450cam and eukaryotic P450s to the extent that the significance level of homology is about four times above what one would expect from randomly generated sequences (15). Since three-dimensional structure is more highly conserved than primary structure, it would be unprecedented if the overall structures bore no relationship to one another. Of the several examples available where the X-ray structure of both a eukaryotic and prokaryotic proteins are known, the overall fold of the polypeptide backbones is always very similar. The one problem with this argument for P450 is that eukaryotic P450s are membrane bound, while P450cam is not. Nevertheless, microsomal P450s contain a hydrophobic N-terminal tail which very likely serves as a membrane anchor, leaving the rest of the enzyme external to the membrane.

In addition to sequence alignments and closely related

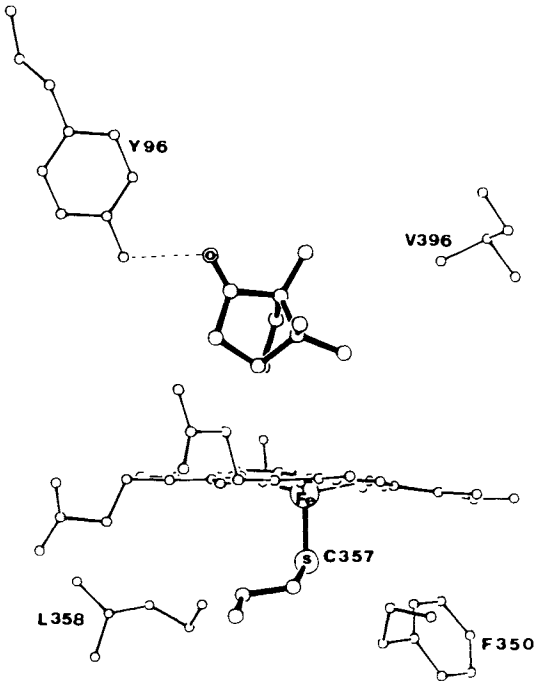

Fig. 2. Edge-on view of the P450cam heme region. The substrate sits directly over and contacts the heme. The camphor forms several hydrophobic contacts with neighboring aliphatic and aromatic side chains in addition to the hydrogen bond with Tyr 96. With substrate bound, Cys357 provides the only axial heme ligand. Cys 357 is close to the surface but is surrounded by highly conserved aliphatic and aromatic side chains. Notice that the iron is displaced from the porphyrin core by about 0.40 Å.

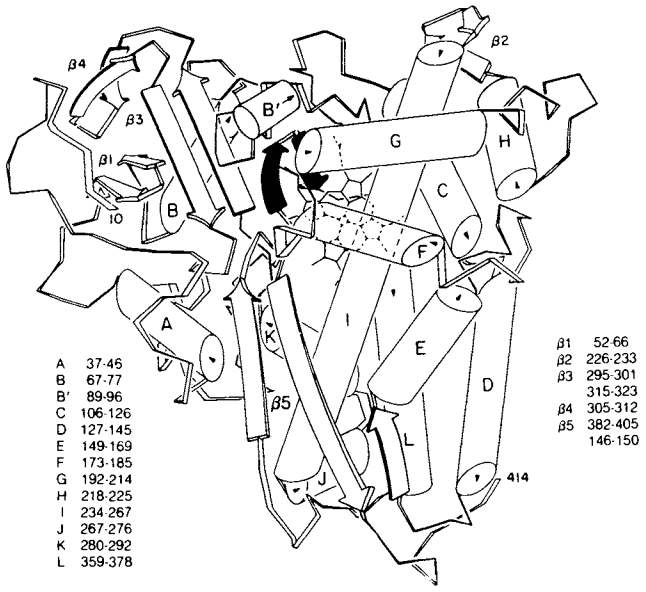

Fig. 3. Schematic representation of the 1.63-Å P450cam structure. Helices are indicated by bars, and beta structure by ribbons. The camphor is hidden from view beneath helix F. The loop connecting helices F and G, the loop connecting the beta strands of β5, and helix B' define the entrance to the substrate pocket. The shaded region highights the β region that contains the axial heme ligand, Cys 357.

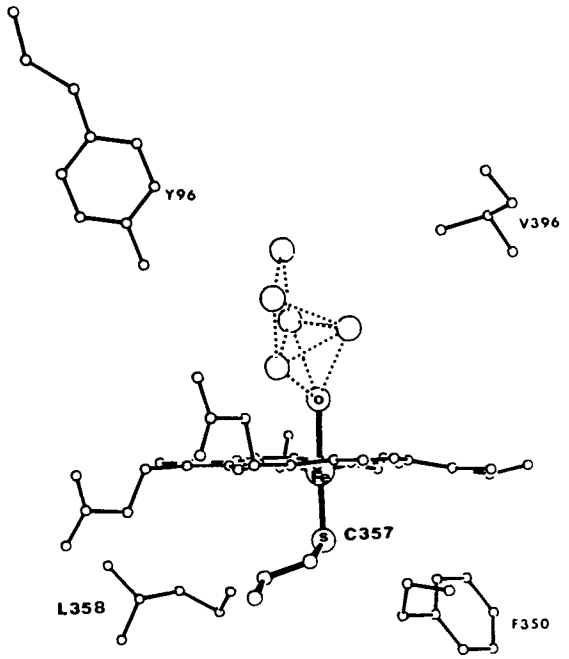

Fig. 4. Edge-on view of the heme in the substrate-free form. A network of hydrogen-bonded water molecules occupies the substrate pocket. One of these water molecules coordinates with the iron atom to give a hexacoordinate, low-spin heme.

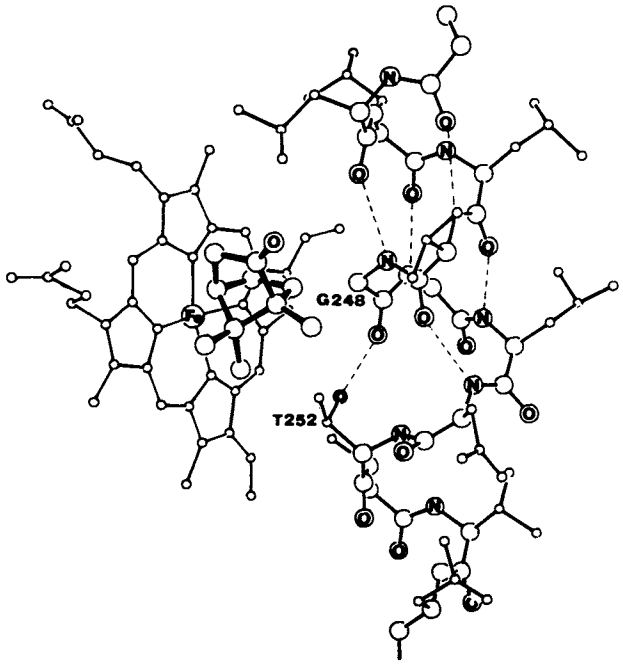

Fig. 5. The highly conserved region of helix I that defines the O_2 binding pocket. The ability of the highly conserved Thr 252 side chain to donate a hydrogen bond to a backbone carbonyl oxygen atom forces a widening in the helix in order to provide a pocket for the O_2 molecule. In a normal alpha helix, the carbonyl oxygen atom of Gly248 would accept a hydrogen bond from the peptide NH of Thr252. Instead, the Gly248 carbonyl oxygen atom hydrogen bonds with the side-chain OH of Thr252. We have postulated that this local disruption in helix I is shared by all P450s and is a requirement in forming the O_2 pocket (13).

spectral properties, there are other functional reasons for favoring a common molecular architecture. We have already seen that the Cys ligand peptide is conserved in all P450s. Less certain is the conservation in the O_2 binding pocket. Yet as we have discussed elsewhere (13), the same functional requirements in O_2 binding and activation in all P450s suggest a similar structure for the oxygen binding pocket. Moreover, sequence homologies extend to the cental region of helix I that in P450cam forms part of the O_2 pocket (Table II). The Gly–Gly or Ala–Gly (positions 248 and 249) sequence is highly conserved, and with only one exception thusfar, Thr252 is conserved. This is of particular interest since helix I undergoes a local distortion and kinking in the vicinity of Thr252 (Fig. 5). The hydrogen bonding capabilities of the Thr252 side-chain hydroxyl group disrupts the local helical hydrogen bonding pattern, forcing a widening of the helix. This opening and bending of helix I provides part of the O_2 pocket. The sequence homology around Thr252 (Table II) indicates a similar O_2 binding pocket in eukaryotic P450s. Further support for this view stems from the suicide substrate inhibition work of Kunze *et al.* (16). These authors used covalent inhibitors of microsomal P450s to map the topography at the active site. The inhibitors covalently attach to the heme, and by careful characterization of the modified heme products, those regions of the heme unavailable for reaction and presumably masked by protein can be accurately determined. The picture that has emerged from these studies is exactly what we see in the P450cam structure. That is, the same pyrrole rings unavailable for reaction in microsomal P450s are masked by helix I in P450cam.

Catalytic Mechanism

Without concern at present for the detailed structure of the catalytic intermediates, the overall P450 reaction can be viewed as occurring in six steps. In the reactions shown below, those intermediates in *italics* are hypothetical and never have been directly observed. The rest form spectrally distinct intermediates.

Table II. Alignment of Distal Helices in Various P450s

P450cam	G	L	L	L	V	G	G	L	D	T_{252}V	V	N	F	L	S	F
P450d (rat)	N	D	I	F	G	A	G	F	E	T_{319}V	T	T	A	I	F	W
P450c (rat)	F	D	L	F	G	A	G	F	D	T_{325}I	T	T	A	I	S	W
P450LM2 (rabbit)	L	S	L	F	F	A	G	T	E	T_{302}T	T	S	T	T	L	R
P450b (rat)	L	S	L	F	F	A	G	T	E	T_{302}T	S	S	T	T	L	R
P450e (rat)	L	S	L	F	F	A	G	T	E	T_{302}T	G	S	T	T	L	R
P450scc (bovine)	T	E	M	L	A	G	G	V	N	T_{329}T	S	M	T	L	Q	W

$$S-Fe^{3+}-OH + RH \rightleftharpoons S-Fe^{3+} \quad RH$$
low spin high spin (1)

$$S-Fe^{3+} + e^- \rightarrow S-Fe^{2+} \quad RH \quad (2)$$

$$S-Fe^{2+} + O_2 \rightarrow S-Fe^{2+}O-O \quad RH \quad (3)$$

$$S-Fe^{2+}O-O + e^- \rightarrow S-Fe^{3+}-O-OH \quad RH \quad (4)$$

$$S-Fe^{3+}-O-OH \quad RH \rightarrow S-Fe^{4+}-O \quad RH \quad (5)$$

$$S-Fe^{4+}-O \quad RH \rightarrow S-Fe^{3+}-OH + R-OH \quad (6)$$

(1) As the crystal structure work has demonstrated, the low-spin substrate-free enzyme contains both a Cys and aqua heme ligands and several solvent molecules in the substrate pocket. We also know that the binding of substrate, RH, is an entropically driven reaction (17) presumably because of the release of active-site water molecules and desolvation of the aliphatic substrate.

(2) The substrate-bound enzyme next is reduced to Fe^{2+} by a flavoprotein in the case of microsomal P450s or an iron–sulfur protein in the case of bacterial P450s including P450cam and mitochrondrial P450s. Reduction of the iron is a requirement for step 3, which is coordination of an O_2 molecule to the heme iron atom.

(3, 4) After oxygenation, a second electron transfer step reduces the iron–oxy species to a hypothetical ferric–peroxy intermediate. The reason for suspecting a peroxy-like intermediate is that in some cases, peroxides can support P450 hydroxylation reactions without the need for the electron transfer steps (18). Additionally, by analogy with the more thoroughly understood peroxidase mechanism, it makes chemical sense to cleave the peroxy O–O bond rather than the more stable O_2 double bond.

(5, 6) After cleavage of the *O–O* bond in a heterolytic reaction resulting in the release of water or hydroxide, the enzyme forms the hypothetical ferryl–oxy, Fe^{4+}–O, intermediate. Here again the analogy is with peroxidase, where the ferryl species has been clearly demonstrated (19,20). The iron-linked "activated" oxygen atom contains only six valence electrons and is a potent electrophile. The oxygen atom is thought to abstract an hydrogen atom from a nearby substrate carbon atom to form a carbon radical that rapidly collapses to the hydroxylated product (21).

There are many gaps in our understanding of the P450 mechanism, especially concerning the nature of the actual hydroxylating species. Also of concern is how the O–O bond is cleaved. While heterolytic fission is favored by analogy with peroxidase and model heme systems, this never has been directly demonstrated. Indeed, Coon and co-workers (22) have shown that P450 will cleave the peroxide bond homolytically to give two oxy radicals but the homolytic process has not been shown to lie along the catalytically competent pathway leading to substrate hydroxylation (23).

The crystal structure of P450cam has shed some light on the mechanism by limiting the possibilities but unfortunately has not led to a detailed stereochemical mechanism as did the crystal structure of cytochrome *c* peroxidase (24). Unlike peroxidases, the P450cam active site does not contain any polar side chains that might play a catalytic role in the O–O bond cleavage reaction. Peroxidase contains His and Arg residues strategically postioned to assist in the transfer of protons and stabilize charge separation in the activated complex, while the oxygen pocket in P450cam is surrounded only by hydrophobic side chains and the substrate molecule (Fig. 6). Thus we can conclude that in sharp contrast to peroxidase, the surrounding protein in P450cam plays an essentially passive role other than providing a hydrophobic environment conducive for substrate binding and oxygenation.

What, then, provides the catalytic push required to

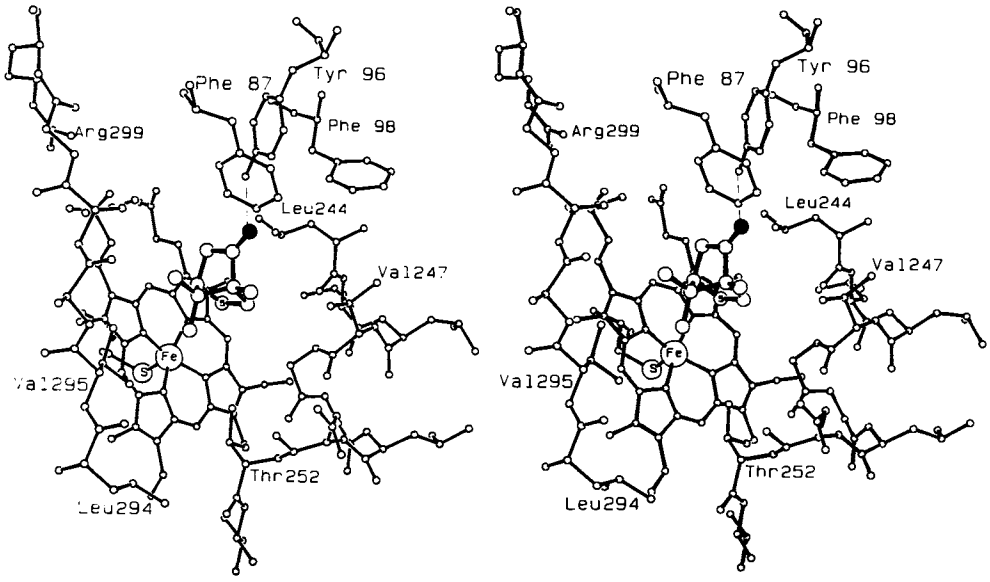

Fig. 6. Stereoscopic view of the P450cam active site. Notice that the camphor and O_2 binding pocket are surrounded by aliphatic and aromatic residues. Unlike peroxidase and catalase, there are no acid–base or polar catalytic residues (His, Arg, etc.) capable of directly assisting in fission of the O–O bond.

achieve cleavage of the O–O bond? As others have argued (25), it must be the Cys ligand. Relative to the His ligand in peroxidase and the Tyr ligand in catalase (26), Cys is a better nucleophile with a lower pK and therefore will ligate to the iron atom as the negatively charged anion. The greater electron donating ability of the Cys sulfur atom apparently is enough to achieve cleavage of the O–O bond without the requirements for polar side chains near the liganded oxygen atoms. The ability of sulfur to form a radical may also play a role in catalysis. What remains unclear is whether the reaction proceeds homolytically to generate two oxygen radicals or heterolytically to release water and retain one oxygen atom, although the heterolytic mechanism appears chemically more reasonable. A second problem is one of acid catalysis. At some point in the reaction cycle, H_2O, HO^-, or HO· is released so protons are required. Unlike other enzyme crystal structures where the acid–base catalysts are usually quite obvious, the source of protons in P450cam is not clear. The obvious choice is water, although we found no ordered water molecules at the active site in the substrate-bound enzyme. Perhaps upon oxygenation the site becomes accessible to a solvent molecule(s). It should be possible to determine the ternary oxy–camphor–P450 complex crystal structure to test this possibility directly.

The crystal structure does allow for a straightforward explanation for the stereoselectivity of the reaction. Gelb *et al.* (27) determined that P450cam hydroxylates camphor to give 5-*exo*-hydroxycamphor as the only product (Fig. 7). As the crystal structure shows, carbon 5 of the camphor molecule is closest to the expected position of the iron-linked oxygen atom and it is the *exo* hydrogen atom that is oriented toward the iron-linked oxygen atom. It therefore appears that the surrounding protein environment rigidly orients the camphor for stereospecific hydroxylation. We might assume that those P450s that do not match the strict stereoselectivity exhibited by P450cam have their substrates bound more loosely at the active site.

The question of stereoselectivity becomes more complex when one looks at the hydroxylation reaction as first

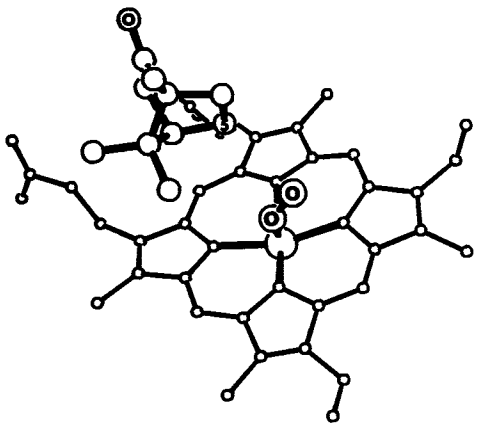

Fig. 7. Hypothetical model of the P450cam–O_2 complex. Notice that C5 of the substrate lies closest to the iron-linked oxygen atom with its *exo* face oriented toward the oxygen atom. Thus, rigid orientation of the camphor molecule relative to the O_2 molecule explains the strict stereoselectivity of the hydroxylation reaction.

the breaking of a C–H bond and then the formation of a C–OH bond. As Groves and co-workers have shown (21), P450-dependent hydroxylation of aliphatic substrates proceeds by, first, abstraction of a hydrogen atom and, then, collapse of the resulting carbon and hydroxy radicals. Thus, in addition to the actual formation of the C–OH bond, hydrogen atom abstraction could involve stereoselectivity. Gelb *et al.* (27) found that either the *exo* or the *endo* hydrogen atoms of the camphor are abstracted, with the *exo* favored, even though, as noted above, hydroxylation proceeds to give only the *exo* product. These results suggest two possibilities. First, the species that abstracts a hydrogen atom and that which forms the C–OH bond are different (27). If this is true, a homolytic mechanism may be involved. Suppose that the O–O bond cleaves to give two oxy radicals, one of which remains bound to the iron while the other is released. The "free" oxy radical might then be able to interact with either the *exo* or the *endo* hydrogen atoms, while the iron-linked oxy radical is restricted to form the *exo* product. The primary concern with such a process is that a highly reactive radical is released in the active site, thus providing the opportunity for reaction with amino acid side chains as well as the substrate. Second, in the more traditional heterolytic, oxene mechanism, only the iron–linked oxygen atom interacts with the substrate. If so, then the substrate must be free to reorient slightly to allow the *endo* hydrogen atom access to the iron-linked oxygen atom.

P450 INHIBITORS

We can define four types of P450 inhibitors: catalytically activated or suicide substrate inhibitors that covalently attach to the heme; reversible inhibitors that mimic substrate binding; those, such as carbon monoxide, that coordinate with the heme iron atom; and those that mimic substrate binding *and* coordinate with the iron atom. We are concerned here with that last type of inhibitor since they represent a class of therapeutic agents that function by blocking P450-dependent hydroxylation reactions usually involving steroid metabolism. Figure 1 shows the structure of various P450 inhibitors that have been used as agrochemicals and chemotherapeutic agents. A particularly important metabolic pathway that can be blocked by P450 inhibitors is ergosterol biosynthesis. Demethylation of lanosterol to ergosterol, the major sterol in fungal membranes, involves P450-catalyzed reactions (11). Disruption of this pathway leads to a buildup of lanosterol and a disruption in fungal membrane function. Perhaps the most well-known anti-P450 therapeutic agent is metyrapone. This agent blocks the P450 11-β-hydroxylase with a K_i of about 10^{-7} M (26). Metyrapone is useful in the treatment of some adrenal tumors that hypersecrete cortisol and in the treatment of Cushing's syndrome, a condition where ACTH is hypersecreted by the pituitary (29). P450s also participate in thromboxane (30), prostaglandin (30), insecticide, and plant growth regulator metabolism (31). Various agents that modify the metabolism of these compounds likely operate by blocking a P450 (11).

All of these inhibitors have in common a heterocyclic nitrogen atom capable of direct coordination with the heme iron atom and a lipophilic region capable of binding in the substrate pocket. The specificity of an inhibitor will be de-

termined by how the lipophilic region interacts with the surrounding protein environment. The variety of molecules metabolized by P450s suggests that the lipophilic substrate pocket is quite diverse in various P450s, which further suggests that designing hydrophobic molecules specific for a particular P450 active-site pocket but attached to a pyridine or imidazole-like ring capable of attachment to the iron atom might be a particularly attractive means for the rational design of therapeutic agents. Critical to this approach, of course, is the requirement for detailed crystallographic information. The P450cam X-ray structure alone clearly is not sufficient to launch a rational drug design program. However, an understanding of inhibitor–P450cam interactions and the design of P450cam inhibitors could provide the basic groundwork and rules, which then could be applied to other, more therapeutically relevant P450s once additional structures become available.

P450cam-Inhibitor Crystal Structures

Toward this end, we recently have determined and analyzed the crystal structure of P450cam inhibited with four compounds: metyrapone and the 1, 2, and 4 isomers of phenylimidazole (32) (Fig. 1). These were chosen because each has been spectrally well characterized (33) and the binding constants are known.

Each of the inhibited structures was highly refined to 2.2-Å resolution and compared in detail to the camphor-bound structure. Figure 8 shows the various inhibited structures superimposed on the camphor–P450cam model. As expected, a heterocyclic nitrogen atom coordinates with the iron atom with the exception of 2-phenylimidazole. With this inhibitor, formation of a N–Fe bond is prevented owing to steric crowding between the inhibitor phenyl group and the heme. 2-Phenylimidazole thus binds in the lopophilic cam-

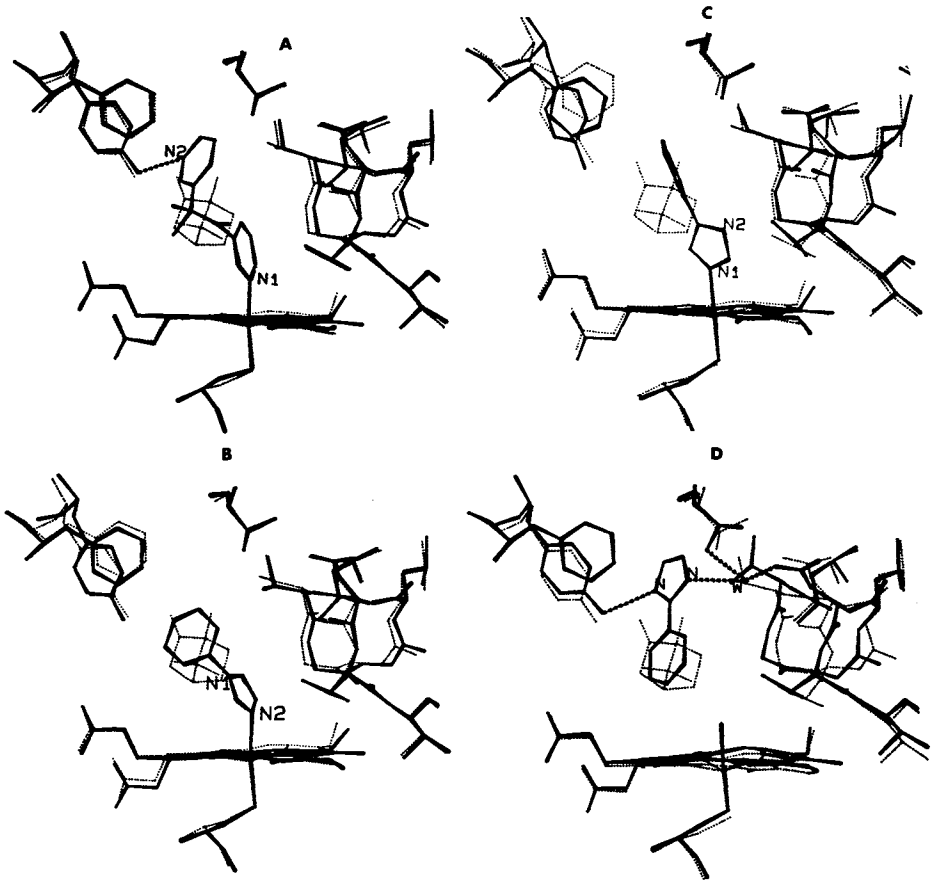

Fig. 8. Models of the P450cam–inhibitor complexes superimposed on the P450cam–camphor-bound structure. Each inhibited structure was refined to a crystallographic R factor of at least 0.18 at 2.2-Å resolution. The dotted lines indicate the native, camphor-bound structure, and the solid lines, inhibited structures. The view is along the helix I axis and shows helix I on the right and the side chains of Phe87 and Tyr96 on the left. (A) Metyrapone; (B) 1-phenylimidazole; (C) 2-phenylimidozole; (D) 4-phenylimidizole. In each structure, Phe87 and Tyr96 adjust to inhibitor binding. In B, C, and E, helix I slides over the heme *away* from the inhibitor, while in D, the helix moves in *toward* the inhibitor. Thus, helix I exhibits a fair range of movement (~3 Å). 2-Phenylimidazole (D) cannot coordinate with the iron atom owing to steric constraints. As a result, a water molecule remains coordinated with the iron atom. A considerable readjustment results when 2-phenylimidazole binds owing to the hydrogen bonding requirements of the imidazole group. A new water molecule (W in D) moves in to hydrogen bond with the inhibitor, while the side chains of Thr185 and Asp251 adjust to help solvate the water molecule. Overall, P450cam exhibits a fair degree of flexibility when inhibitors bind.

phor pocket, while the iron retains an aqua ligand. The iron coordination environment therefore is exactly the same as with substrate-free P450cam.

All of the inhibitors result in significant rearrangement of protein atoms, especially in helix I. The central region of this helix forms part of the O_2 pocket and provides amino acid side chains that contact the substrate molecule. Those inhibitors that coordinate with the iron atom lie close to this segment of helix I, thus requiring a movement of the helix away from the inhibitor. Again, 2-phenylimidazole is an exception. Since this inhibitor does not coordinate with the iron atom, helix I moves *in* toward the inhibitor rather than *away* as with the other three inhibitors. This motion of helix I toward 2-phenylimidazole appears to be required to optimize hydrogen bonding interactions between Asp 251 of helix I, an internal water molecule, and the inhibitor's imidazole group. Movements induced by 2-phenylimidazole also are considerably larger than with the other inhibitors, up to 2.0 Å for some main-chain atoms. Thus, the range of motions available to main-chain atoms in helix I is up to 3–4 Å. This demonstrates a considerable degree of flexibility in a functionally critical part of the P450cam active site. Other regions that exhibit flexibility both in conformation and in dynamical motions, as estimated from crystallographic temperature factors, when inhibitors bind include Phe 87, Tyr 96, and Thr 185, all of which form specific contacts with the substrate. In contrast, the beta 3 segment which forms a specific contact between Val 295 and the substrate remains unchanged.

FUTURE PROSPECTS

The structural changes just described can be utilized in the design of new P450cam inhibitors. For example, we might anticipate that those regions exhibiting conformational and dynamical flexibility would be more "forgiving" upon the formation of an inhibited complex, while the Val 295 region would be much less so. Tyr 96 could be especially important in the design for new inhibitors owing to the hydrogen bonding capability of the side-chain hydroxyl group. Tyr 96 hydrogen bonds with the camphor carbonyl oxygen atom and with a pyridine nitrogen atom of the metyrapone, although the hydrogen bond distance and geometry are much better with camphor. Therefore, it should be relatively straightforward to design a molecule that coordinates with the iron atom, hydrogen bonds with Tyr 96, and forms specific hydrophobic contacts with the rigid Val 295 segment.

The most attractive feature of P450 as a drug target is the broad range of substrate specificities. In contrast to other target enzymes such as dihydrofolate reductase (DHFR) where the receptor pocket exhibits only subtle differences between species (33), P450s exhibit large differences. This should make the design of drug specificity easier to achieve and also minimize a major problem in drug design, host toxicity.

P450cam also should be able to serve as a good model system using molecular dynamics approaches to inhibitor design. Recent advances in coupling statistical mechanical theory with molecular dynamics techniques often called "free-energy perturbation" methods (35,36) should be readily applicable to P450. In this methodology, the "mutation" of one inhibitor with known binding properties into a second inhibitor with unknown binding properties is simulated in a computer. A similar simulation is carried out for the enzyme–inhibitor complex. This approach allows one to calculate the free energy of binding of a new inhibitor to the target enzyme. For such an approach to work, the structures of the known and unknown inhibitors must not differ too much. Herein lies one of the advantages in P450 as a model system since inhibitors with similar structures can have very different affinities for the enzyme. One of the best examples is the isomers of phenylimidazole (Fig. 1) binding to P450cam. The 1-isomer exhibits a dissociation constant of 0.0001 m*M*, while that of the 4 isomer is 0.04 m*M*.

While the above discussion presents an optimistic view of using P450s as targets for rational drug design, realizing this goal depends heavily on determining new crystal structures. The membrane binding properties of eukaryotic P450s may well present a serious obstacle to this goal. Future research would need to focus on the cloning and expression of P450s that lack the N-terminal membrane insertion sequences. Given the large number of P450s for which DNA clones are available, it may be possible to find at least one or two therapeutically relevant eukaryotic P450s that can be expressed as a soluble, active enzyme.

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