The 2.6-Å Crystal Structure of Pseudomonas putida Cytochrome P-450*

Thomas L. Poulos‡‡, Barry C. Finzel‡‡, Irwin C. Gunsalus∥, Gerald C. Wagner∥, and Joseph Kraut**

From the §Protein Engineering Division, Genex Corporation, Gaithersburg, Maryland 20877, the ‡Department of Biochemistry, University of Illinois, Urbana, Illinois 61801, and the **Department of Chemistry B017, University of California at San Diego, La Jolla, California 92039

The crystal structure of Pseudomonas putida cytochrome P-450cam in the ferric, camphor bound form has been determined and partially refined to R = 0.23 at 2.6 Å. The single 414 amino acid polypeptide chain (M_r = 45,000) approximates a triangular prism with a maximum dimension of ~60 Å and a minimum of ~30 Å. Twelve helical segments (A through L) account for ~40% of the structure while antiparallel β pairs account for only ~10%. The unexposed iron protoporphyrin IX is sandwiched between two parallel helices designated the proximal and distal helices. The heme iron atom is pentacoordinate with the axial sulfur ligand provided by Cys 387 which extends from the N-terminal end of the proximal (L) helix.

A substrate molecule, 2-bornanone (camphor), is buried in an internal pocket just above the heme distal surface adjacent to the oxygen binding site. The substrate molecule is held in place by a hydrogen bond between the side chain hydroxyl group of Tyr 96 and chromophore molecule and neighboring aliphatic and aromatic residues. The camphor is oriented such that the exo-surface of C5 would contact an iron bound, "activated" oxygen atom for stereoselective hydroxylation.

Cytochrome P-450 monooxygenases are a widely distributed class of B-type hemoproteins that catalyze a mixed function oxidation of hydrocarbon substrates (1). Utilizing two-electron equivalents from reduced pyridine nucleotides, the P-450 monooxygenases cleave dioxygen into water and a single oxygen atom which is inserted into a hydrocarbon bond. As a group, the P-450 monooxygenases are characterized by a diverse selectivity in substrates and a broad range of functions with toxicological, oncogenic, and regulatory significance (2-4). Despite the breadth of P-450 mediated reactions, a wealth of physical evidence indicates common molecular properties in the catalytic centers of all P-450s (1-4). For example, primary structure data (5-8) reinforces the contention of a similar heme-thiolate (cysteinyl) environment in all P-450-type hemoproteins. As one of the best model systems providing details of the structural and mechanistic homologies (9-12), the microbial cytochrome P-450cam is the only well-known example to be isolated in soluble and crystalline forms.

The camphor 5-exo-monooxygenase (EC 1.14.15.1) from Pseudomonas putida is an inducible, multicomponent P-450 system that incorporates two additional redox proteins, an FAD reductase and a FeS_Cytc redoxin, to transfer electrons from NADH to P-450cam. In a complete, reconstituted system, P-450cam catalyzes the stereospecific 5-exo hydroxylation of the bicyclic terpene camphor which the common soil microbe utilizes as its sole carbon source. As a consequence of the homology in active site properties and the availability of diffraction quality crystals (13), the high resolution x-ray structure of P-450cam should provide a sound molecular basis for understanding the structure and function of many eukaryotic P-450s. We report here the first molecular structure determination of a cytochrome P-450 that reveals the overall fold of P-450cam, identification of the thiolate ligand, and topography of the substrate binding site.

MATERIALS AND METHODS

Crystallization and Data Collection—Crystallization of orthohomobic II P-450cam crystals originally isolated in 1979 (11) was carried out according to our earlier procedure (13). These crystals belong to space group P21212, with unit cell dimensions a = 108.73 Å, b = 104.46 Å, and c = 36.44 Å and one molecule/asymmetric unit. Crystals were stored at 10 °C in an artificial mother liquor consisting of 40% saturated ammonium sulfate, 0.25 M KCl, 1 mM camphor, and 0.05 M potassium phosphate buffer, pH 7.0. Preliminary spectroscopic data (11, 14) obtained from single crystals indicate that P-450cam is in the substrate-bound high-spin ferric state.

X-ray intensity data were collected at ambient temperatures (18 °C) using an Enraf Nonius CAD4 diffractometer. A continuous 2θ/ω scan mode was used. Scan widths ranged from 0.27° to 0.40° at a rate of 0.2° to 0.4° per min. Bijvoet data were collected at ±2°. The extent of x-ray damage was estimated by monitoring the intensities of five standard reflections every 2 h. In most cases data collection was discontinued when a crystal had decayed 20%. Given this criterion, parent crystals lasted for about 60 h while derivative crystals lasted 30 to 48 h.

An empirical background curve was determined for each crystal as a function of 2θ only. Interpolated values from these curves were applied to the intensity data as a background correction. An empirical absorption correction was applied as well by using interpolated values from a 360° + scan (10° intervals) of an intense reflection near χ = 90° (usually an 001 or 000 reflection). Data reduction was carried out...
with the XTAL software system (15).

Heavy Atom Derivatives—Heavy atom derivatives were screened for looking for intensity changes in 15° precession photographs of the h0l and 0kl projections and those exhibiting reproducible intensity changes were considered potential derivatives. The conditions for preparation of derivatives used and a summary of data collection to 3.0 A are summarized in Table I.

Heavy atom positions were located initially by three-dimensional difference Patterson syntheses and derivative Bijvoet difference Pat- terns. After collecting low resolution data (4.0 to 5.0 A) for several derivatives, it was clear that the ethylmercurithiosalicylate derivative gave the most readily interpretable difference Pattersons and also contained a site (site 1) not present in the other derivatives. Therefore, before extending all derivative data to higher resolution, Bijvoet data to 4.0 A were collected for the ethylmercurithiosalicylate derivative, using a total of six crystals, in order to obtain a single isomor- phous replacement phases. Phases derived from the ethylmercurithiosalicylate derivative were then used to locate sites in other derivatives by difference Fourier syntheses. Finally, by correlating cross-difference Fouriers with difference Pattersons, the distribution of sites in each derivative was deduced.

Phase Refinement and Electron Density Maps—Phase calculations and refinement were carried out with the MIR program of the XTAL system (16). The MIR program offers the option of using either the Blow-Crick method (17) or the method of Syusgh (18). A majority of the time the Blow-Crick method was used although both methods gave similar results.

The final statistics of the phase refinement to 3.0 A are shown in Table II. Temperature factors for some of the sites behaved erratically during the course of refinement and, as a result, temperature factors for these troublesome sites were held constant at “reasonable” values and the occupancies and positional parameters alone were allowed to vary. As shown in Table II, only the ethylmercurithiosalicylate and mercuric potassium cyanide derivatives gave good statistics while the remaining derivatives appear to be of marginal value. Nevertheless, inclusion of these poorer derivatives resulted in a higher figure of merit and cleaner electron density maps. Data for the ethylmercurithiosalicylate and mercuric potassium cyanide derivatives were ex- tended to 2.8 A giving a figure of merit of 0.54 at 2.8 A. Prior to crystallographic refinement of the structure, data were obtained from two additional parent crystals giving a total of 12,948 unique reflec- tions to 2.6 A for the native data set.

Electron density maps were computed on a 1.5-Å grid and displayed on plastic sheets using a 4.0-Å/cm scale. Fitting of the known sequence (8) was carried out initially at the University of California, San Diego, using an Evans and Sutherland Picture System II and locally developed software while at Genex, an Evans and Sutherland PS300 color graphics system supporting FRODO (19) was used.

Crystallographic Refinement—The restrained parameter least squares refinement of Hendrickson and Konnert (20) modified by one of us (B. C. F.) to employ the fast Fourier transform algorithm of Agarwal (21) was used. Computed and multiple isomorphous phase probability distributions were combined to generate a new set of phases as described in Refs. 22 and 23. We emphasize that phase combination proved to be extremely important in refining the structure since, in the early stages of refinement, electron density maps computed with combined phases contained considerably more useful information than maps computed using heavy atom or calculated phases. Between 2.8 and 2.6 A, phases were obtained from computed structure factors since there were no multiple isomorphous replacement phases in this resolution range.

RESULTS AND DISCUSSION

Solution of the Structure—An initial 3.0-A electron density map was sufficiently clear to outline a single P-450cam molecule, locate the heme, and determine the direction of most helical segments. Nevertheless, we were unable to derive an uninterrupted tracing of the polypeptide chain. In retrospect, this difficulty was the result of a break in the polypeptide chain electron density between residues 216 and 226 in addition to distortions typical in the electron density near heavy atom binding sites. Several unsuccessful attempts were made to fit the sequence to the electron density map but only the distal helix (helix I, Figs. 1A and 2) could be located with confidence. It was evident that a solution of the structure would be extremely difficult without first improving the electron density map.

The electron density was improved significantly by a phase combination procedure (22-24). First, a trial structure was derived by emphasizing the fit of idealized secondary structure to the electron density map and wherever possible side chains were included. In fitting side chains, emphasis was placed on the size, shape, and location of electron density envelopes rather than adhering strictly to the sequence. No attempt was made to model ambiguous connections between segments. The resulting trial structure contained about 80% of the expected nonhydrogen protein atoms. We emphasize that this initial structure was designed to serve as a source of computed phases and was by no means the correct structure.

Eight cycles of restrained least squares refinement lowered R from 0.59 to 0.48. Computed and heavy atom phases were combined giving an overall figure of merit for the combined phases = 0.63 to 2.8 A compared with 0.54 for the 2.8-A multiple isomorphous replacement phases. Most importantly, 2Fo-Fc, or Fo electron density maps computed with combined phases enabled more of the sequence to be located correctly. Four additional rounds of model building, refinement, and phase combination lowered R to 0.34. At this stage, we dis-

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1The abbreviations used are: Fo, observed structure factors; Fc, calculated structure factors; R = \[ \frac{2}{\sum |F_o|/ \sum |F_o + F_c|} \].
covered an error in the tracing of the polypeptide chain necessitating a complete reinterpretation of the \( R = 0.34 \) electron density map. The new trial structure was subjected to an additional 52 cycles of refinement interrupted twice after cycles 18 and 35 for major manual readjustments of the model. Temperature factors were not refined nor have solvent molecules been included since at 2.6 \( \AA \) we feel there are too few data to justify the inclusion of these additional parameters. The final crystallographic \( R = 0.23 \) for 10,027 reflections \( (U \geq 2 \sigma) \). Other pertinent refinement statistics are summarized in Table III.

During the latter stages of refinement, the \( P-450\text{cam} \) DNA sequence became available. The predicted protein sequence indicates that a dipeptide, Trp-Thr, should be inserted after Val 54 and that residue 361 (formerly 359), is His, not Ser. Incorporation of these changes into our atomic model considerably improved the fit to the electron density map and buried some of our confusion in interpreting a sharp reversal in the polypeptide chain between residues 55 and 63.

In our current refined model, the entire primary sequence can be accounted for in the electron density map with the exception of residues 1–9. Electron density for residues 1–9 is present but not readily interpretable, indicating that these residues are relatively disordered.

**Overall Topography**—\( P-450\text{cam} \) resembles a triangular prism \( \sim 30 \) \( \AA \) thick with sides 55 to 60 \( \AA \). Twelve helical segments dominate the structure (Figs. 1 and 2) and account for about 40% of the residues. Approximately 10% of the structure is divided into five antiparallel \( \beta \) pairs while there is no parallel \( \beta \) or extended sheet structure. Helices E, F, G, and I form an antiparallel Greek key helical bundle, a common structural motif found in many helical proteins (25). Helices I, L, and D are in approximate parallel alignment. Residues found in a helical conformation are about equally divided between the N- and C-terminal halves of the sequence although a majority of helical structure is clustered on the right half of the protein (refer to Figs. 1A and 2). Three of the five antiparallel \( \beta \) pairs are located in the C-terminal half of the molecule. As a result, \( P-450\text{cam} \) does not fold into an N- and C-terminal domain as do many other enzymes. Instead, the polypeptide chain begins on the left side (Figs. 1 and 2), crosses over to the right where most of the helices are found, over to the left, and once more to the right where Val 414, the C terminus, is situated near helix D. Although \( P-450\text{cam} \) contains no hinge or cleft which defines separate lobes, the molecule is divided into a helical rich domain (right half, Figs. 1A and 2) containing helices C–L and a helical poor domain containing helices A and B and \( \beta \) 1, 3, and 4 (left half, Figs. 1 and 2).

**Heme Environment**—The heme disc is embedded between the proximal (helix L) and distal (helix I) hemes. Such an arrangement is a common feature in many heme proteins (26). The histidine ligand \( c \)-peroxidase (27), the histidine ligand extends from the C-terminal end of the proximal helix. In contrast, the heme thiolate ligand extends from the N-terminal end of the proximal helix, while in the globins (26) and cytochrome \( c \) peroxidase (27), the histidine ligand extends from the C-terminal end of the proximal helix.

Except for the heme propionates which interact with Arg 112, Arg 299, and His 355, the heme is surrounded by apolar

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2 S. Sligar, personal communication.
residues. Residues interacting with the heme edges extend from one strand of $\beta$ 3 (residues 294 to 299) and helix C. The heme is completely surrounded with no edge accessible to the molecular surface. The closest approach of the heme to the surface occurs at the proximal face, a distance of about 8 Å, although residues 350 to 358 mask the proximal surface.

A buried heme is also found in the other known heme enzyme structures, catalase (28) and cytochrome $c$ peroxidase (27), but sharply contrasts with the exposed heme edges found in the cytochromes (29). Such differences in heme accessibility have significant implications regarding electron transfer mechanisms. In both P-450 cam and cytochrome $c$ peroxidase, the heme is buried and cannot accept electrons from macromolecular electron transfer donors via direct contact between prosthetic groups while heme edge contact between cytochromes is possible. Therefore, in both cytochrome $c$ peroxidase and P-450cam, an incoming electron must encounter protein groups. Hypothetical modeling of the peroxidase-cytochrome $c$ complex (30), which now has received experimental support (31–34), indicates that a specific hydrogen bonding path directs the transfer of an electron. We might anticipate parallels for P-450cam although our understanding of the P-450cam-putidaredoxin system is not as well advanced since the structure of putidaredoxin is unknown. However, we do observe a possibly important difference between cytochrome $c$ peroxidase and P-450cam. Unlike the peroxidase and cytochrome $c$, P-450cam appears not to exhibit an asymmetric distribution of charged groups on the molecular surface that might serve as an obvious ionic or electrostatic recognition site for putidaredoxin.

**Axial Heme Ligands**—As shown in Figs. 3–5, the axial thiolate ligand (9–12, 35–39) is provided by the side chain of Cys 357 which extends from the N-terminal end of helix L, although the Cys 357 peptide is not in a helical conformation. Comparison of several P-450 sequences (Table IV) reveals that the most highly conserved region coincides with the Cys
**FIG. 2.** Schematic diagram of P-450cam in the same orientation as in Fig. 1A. Helices are represented as rods and β pairs as flat arrows. The inset lists residues in a helical and antiparallel β conformation.

### Table III

Summary of least squares refinement parameters

<table>
<thead>
<tr>
<th>Reflections to 2.6 Å</th>
<th>12,948</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reflections ≥ twice background</td>
<td>10,027</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Distances</th>
<th>Target σ</th>
<th>Final model</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>0.030</td>
<td>0.037</td>
</tr>
<tr>
<td>1-3</td>
<td>0.040</td>
<td>0.037</td>
</tr>
<tr>
<td>1-4</td>
<td>0.050</td>
<td>0.072</td>
</tr>
</tbody>
</table>

| Planes | Peptides | 0.040 | 0.046 |
|        | Other    | 0.040 | 0.049 |

| Chiral volumes | 0.300 | 0.506 |

| Nonbonded contacts | 0.500 | 0.288 |
|                   | 0.500 | 0.288 |

357 peptide thus supporting the conclusion (5, 6) that the eukaryotic homologue to Cys 357 provides the thiolate ligand in eukaryotic P-450s.

Two of the conserved residues near Cys 357 provide hydrophobic contacts with the heme as depicted in Fig. 3. Phe 350 and Leu 358 bracket Cys 357 and contact the heme proximal surface and together with Gln 360 form a pocket for the thiolate ligand. Examination of Table IV shows that Phe 350 is invariant while Leu 358 is either Leu or Ile in microsomal P-450s and Val in the mitochondrial cholesterol side chain cleavage enzyme, P-450scc. In addition, His 355 (Fig. 3) is in position to hydrogen bond with one of the heme propionates and the corresponding residue in eukaryotic P-450s is an Arg, a functionally conservative change. An invariant glycine (Gly 353) initiates a type I turn, and a second invariant glycine, Gly 359, terminates the N-terminal end of the proximal helix just below the heme where a Gly is required owing to the close approach of the polypeptide chain to the heme. Such striking homologies indicate a similar if not identical three-dimensional arrangement of residues on the proximal heme surface in all P-450s.

No electron density appears at the axial oxygen coordination position indicating that in the ferric, camphor bound state, P-450cam is pentacoordinate in agreement with the ENDOR data of Lobrutto et al. (38). However, there is a pocket large enough for an O₂ molecule which we anticipate is occupied by loosely bound or disordered solvent molecules not visible in our current electron density maps. We can only speculate with regard to the sixth ligand, if any, in the camphor-free, low-spin state. There are no neighboring oxygen or nitrogen containing side chains capable of interacting directly with the iron atom without an unlikely and large disruption of secondary structure. Alternatively, we favor a water or hydroxide ligand which is consistent with the spectroscopic data (42, 45).

**Camphor Binding Site**—Our initial 2.8-Å multiple isomorphous replacement electron density map exhibited an isolated lobe of density just above the heme distal surface, precisely the correct size for a camphor molecule. At the time we could not be certain how the camphor should be oriented so we chose not to model a substrate molecule during the course of refinement. At the end of our current refinement efforts, however, it became clear that the initial estimate of how the camphor should be oriented was correct.

Fig. 4 demonstrates the fit of a camphor molecule, the
Fig. 3. Stereoscopic model of P-450cam in the immediate vicinity of the thiolate ligand, Cys 357.

Fig. 4. Stereoscopic view of the 2F₀-Fc electron density map using combined phases demonstrating the fit of a camphor (CAM) molecule. Also shown are the heme, Tyr 96, and the region near Cys 357. A camphor molecule was not included in the structure factor calculation used to generate computed phases.

heme, Tyr 96, and a section of the molecule containing Cys 357 to our most recent 2F₀-Fc electron density map. A thin finger of electron density connects the camphor molecule with the hydroxyl group of Tyr 96. We interpret this feature as a hydrogen bond between Tyr 96 and the only atom in camphor capable of hydrogen bonding, the carbonyl oxygen atom. Given this restriction, a camphor molecule fits into the electron density optimally in only one way, as shown in Fig. 4. We emphasize the excellent fit of His 361 to the electron density since residue 361 was initially identified as Ser (8) while the DNA sequence indicates His.

Fig. 5 depicts the overall topography in the substrate binding pocket. The substrate molecule sits about 4 Å above pyrrole ring A, directly adjacent to the oxygen binding site. In addition to the Tyr 96 hydrogen bond, the camphor molecule is oriented by a series of complementary hydrophobic contacts with neighboring residues. Particularly noteworthy are Phe 87, Leu 244, Val 247, CG2 of Thr 252, and Val 295. Not shown in Fig. 5 for clarity are Ile 385 and Phe 193 which form a cap over the camphor molecule.

The camphor molecule is buried with no readily apparent path from the enzyme molecular surface to the substrate pocket. However, three sharp reversals in the polypeptide chain centered on residues 88, 185, and 395 are juxtaposed at the molecular surface to form a distinct depression and small opening above the camphor molecule. Even so, examination of the computed van der Waals surface on a computer graphics system shows that this opening cannot accommodate a camphor molecule so if this depression provides an entrance, it must be flexible. Flexibility in the heme pocket actually is expected because P-450cam binds a variety of molecules larger than camphor such as metyrapone and phenylimidazole (46).

Comparisons with Eukaryotic P-450s—First we consider the distal side and ask whether or not the environment in the immediate vicinity of the oxygen ligand is conserved. One section we might anticipate to be conserved is the 244-252 part of the distal helix since this segment sits directly above the heme surface and provides the closest anticipated contact points for an oxygen molecule. Sequence alignments reproduced in Table V indicate a similar apolar patch in the distal helix. Residues 244-249 (Cam numbering) form the principal distal helix-heme contact points and as such must be apolar. Note, too, that residues 248-249 are always Gly-Gly or Ala-Gly. A large side chain at position 249 could not be tolerated owing to the close approach of Gly 249 to the distal heme surface. In addition, the normal helical hydrogen bonding pattern is disrupted beginning with Gly 248 such that the side chain oxygen atom of Thr 252 donates a hydrogen bond to the peptide carbonyl oxygen atom of Gly 248. The normal helical hydrogen bonding pattern resumes at residue 255.
P-450cam Crystal Structure

![Structure Diagram]

**FIG. 5. Stereoscopic view of the camphor binding site.** Pyrrole ring nitrogens and C5 of the camphor molecule are labeled.

**TABLE IV**

*Alignment of sequences in the vicinity of the thiolate ligand*

The current status of available P-450 sequence data is reviewed in Refs. 56–59.

<table>
<thead>
<tr>
<th>P-450cam</th>
<th>FGHGSHLC357LGQHLAR</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-450d (rat)</td>
<td>FGLGKRRC456IGIKPAK</td>
<td>40</td>
</tr>
<tr>
<td>P-450c (rat)</td>
<td>FGLGKRRC461IGETIRR</td>
<td>41, 44</td>
</tr>
<tr>
<td>P-450LM2 (rabbit)</td>
<td>FSLGKRIC461LGEQIAV</td>
<td>57</td>
</tr>
<tr>
<td>P-450b,e (rat)</td>
<td>FSTGKRIC461MGEQIAV</td>
<td>5, 40</td>
</tr>
<tr>
<td>P-450cc (bovine)</td>
<td>FGWGVRQC466VGRGIAE</td>
<td>40, 44</td>
</tr>
</tbody>
</table>

Disruption or termination of helices by threonine or serine is a common protein structural feature. That Thr 252 is conserved in all P-450s for which sequence data are available suggests that the local deformation of the distal helix just above the heme is an important feature shared by many if not all P-450s.

Even though the overall apolarity of the 244–249 segment is conserved, the specific amino acids are not. However, two of these residues, Leu 244 and Val 247, contact the substrate molecule (see below) and are, therefore, just those positions which should change from one P-450 to the other as substrate specificity changes.

Asp 251 stands out as the only ionic residue in the 244–252 part of the distal helix and as such deserves further discussion, especially since an acidic residue at this position is highly conserved. Asp 251 points away from the heme to form an internal ion pair with Arg 186 which probably adds additional stability to the distorted region of the distal helix. Sequence alignments (47) suggest that Arg or Lys may occur at position 186 in eukaryotic P-450s.

Finally, the elegant suicide substrate work of Kunze *et al.* (48) argues for a common topography near the oxygen pocket in both microsomal and bacterial P-450s. These investigators used a series of alkenes and alkynes that operate as suicide substrates by alkylation of a pyrrole ring nitrogen. By varying the size of the inhibitor used and by dissecting the stereochemistry of the products, a topographical picture of the microsomal P-450 active site emerged which is remarkably similar to what we find in the P-450cam X-ray structure. That is, pyrrole ring A is open to substrate binding but ring C is blocked by the distal helix. Coupled with the above discussion on the distal helix, these results imply a very similar topography shared by all P-450s in the immediate vicinity of the oxygen binding pocket.

Next we consider those regions of the substrate pocket most likely to vary between P-450s. As we discussed in the preceding section, the distal helix is very likely to be similarly positioned in all P-450s and only a few side chains directly contacting the substrate will change. On the other hand, we anticipate that those regions most likely to exhibit the highest

**TABLE V**

*Alignment of distal helices in various P-450s*

The Hydrophobic stretch running over the distal heme surface is underlined and residues marked with an (*) contact the camphor molecule.

<table>
<thead>
<tr>
<th>P-450cam</th>
<th>GLLVTGLD725VNFSL</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-450d (rat)</td>
<td>NDIIFGAFET319VTTAIF</td>
<td>40</td>
</tr>
<tr>
<td>P-450c (rat)</td>
<td>PDLFGAGFDT325TSAIS</td>
<td>41, 44</td>
</tr>
<tr>
<td>P-450LM2 (rabbit)</td>
<td>LSLFAGET302TSSTTTL</td>
<td>57</td>
</tr>
<tr>
<td>P-450b (rat)</td>
<td>LSLFAGET302TSSTTTL</td>
<td>5</td>
</tr>
<tr>
<td>P-450e (rat)</td>
<td>LSLFAGET302TSSTTTL</td>
<td>40</td>
</tr>
<tr>
<td>P-455cc (bovine)</td>
<td>TEMLAGGNT328TSMTLQW</td>
<td>40, 44</td>
</tr>
</tbody>
</table>
degree of variability will occur in the vicinity of Tyr 96 and Val 295. Tyr 96 provides a specific hydrogen bond presumed to be unique to P-450cam. Val 295 and its associated segment of polypeptide chain (β 9) restrict the size of the substrate capable of binding in the pocket. In order to accommodate larger substrates common in eukaryotic P-450s, a rearrangement in the vicinity of Val 295 must occur, of course, that the distal helix remains fixed. Furthermore, sequence alignments (47) indicate that neither the Tyr 96 nor Val 295 regions are conserved so we might look to these regions as the primary variable sites controlling substrate specificity. This is a reasonable working hypothesis based on structural considerations as well as sequence alignments. Note that the main region which we postulate to control substrate specificity, β 3, is confined to the helical poor domain (left halves, Figs. 1A and 2). Since helices are rather rigid structures with specific packing requirements, the helical rich domain may be relatively inflexible and less likely to change significantly from one P-450 to the next. In contrast, the helical poor domain should be more readily susceptible to significant changes in polypeptide topography owing both to the low helical content and lack of extended secondary structure.

Mechanistic Implications—We conclude by examining what insights the x-ray structure provides with regard to the oxygen activating and hydroxylation steps in the catalytic cycle. Camphor hydroxylation is a regio- and stereoselective process giving 5 exo-hydroxy camphor as the only product (49, 55). As shown in Fig. 5, the exo-surface of the C5 carbon atom is oriented toward the oxygen site such that an activated iron bound oxygen atom would contact C5 on the exo-surface only. It therefore appears that the set of complementary hydrophobic substrate-protein interactions and the Tyr 96-camphor hydroxyl-bond orient the substrate such that the C5 exo-position is closest to the iron bound oxygen atom. The P-450cam structure also provides a simple explanation for why a substrate analogue, 5,5-difluorocamphor, containing two fluorine atoms on C5 is hydroxylated exclusively at C9 (50). C9 is one of the methyl groups extending from the bridging methylene carbon atom and besides C5, C9 is the closest camphor atom to the anticipated oxygen binding site. Therefore only a slight rotation of the camphor molecule without significant disruption of specific protein-substrate contact points is required to bring C9 into direct contact with an iron bound oxygen atom. The actual oxygen activation step is less clear. Three general mechanisms have been proposed which result in activated iron bound oxygen atom. There are two essentially new data sets obtained from two crystals using a Xentronics area detector and a rotating anode x-ray source. Analysis of the refined 1.7-A structure confirms our conclusions based on the 2.6-A structure. α-Carbon coordinates have been deposited with the Brookhaven Protein Data Bank.

Acknowledgment—Two of us (T. L. P. and B. C. F.) would like to thank Dr. Robert Ladner for implementation of graphics and plotting software at Genex.

Addendum—Since submitting this manuscript, we have refined the P-450cam crystal structure to R = 0.19 at 1.7-A resolution using a completely new data set obtained from two crystals using a Xentronics area detector and a rotating anode x-ray source. Analysis of the refined 1.7-A structure confirms our conclusions based on the 2.6-A structure. α-Carbon coordinates have been deposited with the Brookhaven Protein Data Bank.

REFERENCES