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Crystallization and preliminary x-ray diffraction analysis of $P450_{terp}$ and the hemoprotein domain of $P450_{BM-3}$, enzymes belonging to two distinct classes of the cytochrome P450 superfamily

(P450 structure/bacterial P450)

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ABSTRACT **Cytochromes P450 are members of a superfamily of hemoproteins that are involved in the metabolism of various physiologic and xenobiotic organic compounds. This superfamily of proteins can be divided into two classes based on the electron donor proximal to the P450: an iron–sulfur protein for class I P450s or a flavoprotein for class II. The only known tertiary structure of any of the cytochromes P450 is that of $P450_{cam}$, a class I soluble enzyme isolated from *Pseudomonas putida* (product of the *CYP101* gene). To understand the details of the structure–function relationships within and between the two classes, structural studies on additional cytochromes P450 are crucial. We report here characterization of the crystal forms of two soluble, bacterial enzymes: cytochrome $P450_{terp}$ [class I enzyme from a *Pseudomonas* species (product of *CYP108* gene)] and the hemoprotein domain of cytochrome $P450_{BM-3}$ [class II enzyme from *Bacillus megaterium* (product of the *CYP102* gene)]. The crystals of cytochrome $P450_{terp}$ are hexagonal and belong to the space group $P6_122$ (or its enantiomorph, $P6_522$) with unit cell dimensions $a = b = 68.9$ Å and $c = 458.7$ Å. The crystals of the hemoprotein domain of cytochrome $P450_{BM-3}$ are monoclinic and belong to the space group $P2_1$ with unit cell dimensions $a = 59.4$ Å, $b = 154.0$ Å, $c = 62.2$ Å, and $\beta = 94.7°$. Diffraction data for the crystals of these two proteins were obtained to a resolution better than 2.2 Å. Assuming the presence of two molecules in the asymmetric unit for the hemoprotein domain of $P450_{BM-3}$ and one molecule for $P450_{terp}$, the calculated values of V_m are 2.6 and 3.3 Å^3/Da, respectively.**

Cytochromes P450 are members of a superfamily of *b*-type heme proteins that catalyze a variety of oxidative reactions in the metabolism of endogenous and exogenous hydrophobic substrates (1–3). These enzymes are isolated from organisms ranging from bacteria to humans (4–6), and their physiological effects cover the spectrum from being required for normal growth and differentiation to the activation of carcinogenic compounds. Cytochromes P450 can be divided into two classes based on their proximal electron donors: class I requiring a FAD-containing reductase and an iron–sulfur protein and class II requiring a reductase that contains both FAD and FMN. Eukaryotic forms of these enzymes are located in the membranes of mitochondria (class I) and the endoplasmic reticulum (class II). In contrast, prokaryotic cytochromes P450 in general are soluble.

The most comprehensively studied of any of these is soluble $P450_{cam}$,¶ a member of class I, isolated from *Pseudomonas putida* (refs. 7–11). In efforts to better understand this superfamily of enzymes, many soluble bacterial cytochromes P450 have been isolated; the genes of at least 11 of these have been cloned, and their nucleotide sequences have been determined (for a review, see ref. 12). Like the mitochondrial enzymes, the bacterial systems, in general, require three components for performing the oxidative reactions. We have isolated a soluble P450 ($P450_{terp}$) from a strain of *Pseudomonas* that was selected by culture enrichment techniques based on the ability to grow with α-terpineol as the sole carbon source (13). Like other class I enzymes, $P450_{terp}$ requires a FAD-containing reductase and an iron–sulfur protein to complete the electron-transfer system essential for the monooxygenation of the hydrocarbon substrate, α-terpineol. The only bacterial P450 that is known to utilize a FAD- and FMN-containing flavoprotein reductase is $P450_{BM-3}$ (class II). This flavocytochrome (M_r 120,000) is a soluble monooxygenase from *Bacillus megaterium* (ATCC 14581) that catalyzes the hydroxylation and epoxidation of several fatty acid substrates (12). The gene encoding this catalytically self-sufficient single polypeptide was genetically engineered, resulting in the separate expression of the two functional domains: the NH_2-terminal domain (M_r 55,000) that contains the heme and functions as the monooxygenase and the COOH-terminal domain (M_r 66,000) that contains both FAD and FMN and functions as a NADPH:cytochrome reductase (14, 38). Comparison of the amino acid sequences of the individual domains of $P450_{BM-3}$ to the sequences of other cytochromes P450 and reductases demonstrated that this protein is more related to class II than to class I enzymes (12, 15). Studies in our laboratory to establish the relationship of this soluble, bacterial enzyme to eukaryotic class II enzymes have included the characterization of the metabolites formed (16, 17), preparation of the individual domains of the enzyme by recombinant DNA methods (14, 38), and reconstitution of the monooxygenase activity of cytochrome $P450_{BM-3}$ by utilizing the individual domains (38).

While a protein can be rather unambiguously identified as a P450 based on its physicochemical properties and conserved primary structure motifs, the structure–function relationships among the cytochromes P450 remain equivocal. In spite of the isolation of many prokaryotic and eukaryotic P450 enzymes in apparently homogeneous forms, the atomic structure of only one of these is available, that of $P450_{cam}$ (18–21). The lack of availability of other tertiary structures has hindered progress in understanding the substrate speci-

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Abbreviations: $P450_{cam}$, soluble cytochrome P450 isolated from *Pseudomonas putida* (the product of *CYP101* gene); $P450_{BM-3}$, soluble cytochrome P450 isolated from *Bacillus megaterium* (the product of *CYP102* gene); $P450_{terp}$, soluble cytochrome P450 isolated from a *Pseudomonas* species (the product of *CYP108* gene); PEG, polyethylene glycol.

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¶The P450 nomenclature is adapted from that of Nebert *et al.* (4).

ficity, interaction with electron-transfer partners, and the basis for the catalysis of similar chemical reactions by distantly related enzymes, etc. To obtain answers for some of the above questions, models for the eukaryotic enzymes have been proposed based on the available structure (22–25). However, the validity of utilizing the structure of $P450_{cam}$ as a general model for all cytochromes P450 has not been completely established (26). Thus, determination of the tertiary structures of additional enzymes, representing both the class I and class II enzymes, is critical to establish the common structural features of these hemoproteins. To this end, we report here the crystallization and preliminary x-ray diffraction analysis of $P450_{terp}$ and the hemoprotein domain of $P450_{BM-3}$, enzymes belonging to the two classes of the P450 superfamily.

MATERIALS AND METHODS

Materials. All chemicals were of ACS grade or better and were used without further purification.

The plasmid BM_{3-2A} (15), containing the $P450_{BM-3}$-encoding region, was provided by A. J. Fulco (Department of Biological Chemistry, University of California, Los Angeles). A recombinant plasmid that contained the region encoding the hemoprotein domain of $P450_{BM-3}$ was constructed by removing the excess of the 5′ flanking region and mutating the codon for Lys-472 to a stop codon. By utilizing the expression procedures developed for $P450_{BM-3}$ (16) and its flavoprotein domain (14), the hemoprotein domain was overexpressed at a level of ≈30% of the *Escherichia coli* soluble proteins. The heterologously produced protein was purified to homogeneity; its properties were similar to those of the native holoenzyme. The individually isolated domains of $P450_{BM-3}$ were utilized to reconstitute the fatty acid hydroxylation activity of the native enzyme (17). Details pertaining to the recombinant DNA methods, expression, purification and properties of the protein, and the ability of the hemoprotein domain to accept electrons from its reductase counterpart are described elsewhere (38).

$P450_{terp}$ was purified and characterized from a pseudomonad that had been isolated by culture enrichment techniques from a swamp in North Dallas for its ability to utilize α-terpineol as a carbon and energy source (13). The purification of this enzyme and the cloning and sequencing of its operon will be published elsewhere (39). Briefly, the bacterial cells were broken by sonication, and the cellular debris was removed by ultracentrifugation. After ammonium sulfate fractionation, the $P450_{terp}$-containing fraction was chromatographed on DE-52 cellulose, Sephadex G-100, DEAE-Sephadex, and finally, another Sephadex G-100 column.

The final preparations of both of these proteins gave a single band on SDS/polyacrylamide gel electrophoresis when the gels were stained with silver. The buffers in the purified proteins were exchanged for 50 mM potassium phosphate (pH 7.4).

Crystallization of $P450_{BM-3}$. The initial crystallization of the hemoprotein domain of $P450_{BM-3}$ was carried out with the hanging-drop vapor-diffusion technique (27) in 24-well Linbro cell culture plates (Flow Laboratories). Typically, the protein solution (5 mM; ≈275 mg of protein per ml) in 50 mM potassium phosphate (pH 7.4) was mixed with an equal volume of a precipitant solution containing 100 mM Pipes, 20% (wt/vol) polyethylene glycol (PEG) 8000, 15 mM dithiothreitol, and 40 mM $MgSO_4$ at pH 6.8. Incubation overnight at 4°C produced crystals.

Crystallization of $P450_{terp}$. Crystals were grown by the hanging- and sitting-drop methods (27). Refinement of the crystallization conditions was accomplished by the streak seeding technique (28) utilizing a cat whisker. Drops were preequilibrated for 48 hr prior to seeding. Hexagonal crystals were obtained by mixing protein solution [1 mM; ≈50 mg of protein per ml in potassium phosphate buffer (pH 7.4)] with precipitant solution (100 mM Pipes buffer/14–16% PEG 12,000/15 mM dithiothreitol/40 mM $MgSO_4$, pH 6.4) at a ratio of 3:5. Incubation for 2 days at 4°C produced crystals.

X-Ray Diffraction Data Collection and Analysis. Crystals were mounted in thin-walled glass capillary tubes, and precession and still x-ray diffraction photographs were taken by using nickel-filtered CuK_α radiation from a Rigaku Rotaflex rotating anode generator (RU-300) at 50 kV and 108 mA with an Enraf-Nonius precession camera. In the case of $P450_{terp}$, to visualize unambiguously the spacing of the reflections along the c^* axis, a 0.1-mm double pinhole collimator was used.

Diffraction data for the hemoprotein domain of $P450_{BM-3}$ were measured at room temperature by ω scans on dual multiwire area detectors of Xuong/Hamlin design (29) with graphite monochromated CuK_α x-rays generated by a Rigaku RU-200 rotating anode generator operating at 50 kV and 108 mA. Data were collected in 0.1° frames at a rate of 20 or 30 sec per frame, with the crystal-to-detector distances of 720 and 780 mm. Detectors were attached to helium cones to minimize air absorption. Data collection, reduction, merging, and scaling of the symmetry-related intensities were accomplished with software by Howard *et al.* (30).

Diffraction data for $P450_{terp}$ were collected at the Cornell High Energy Synchrotron Source (CHESS) F1 station by utilizing a wavelength of 0.91 Å collimated with a 0.1-mm collimator. The data were collected by the oscillation method with Kodak storage phosphors as detector. The crystals were aligned with the c axis along the capillary–spindle axis to permit an oscillation of 2.5° per data frame. Three data sets, each from a single crystal, were collected. A 30° data set was acquired with the x-ray beam at detector center and a crystal-to-detector distance of 350 mm. Data for the second and third crystals were obtained with the detector translated 80 mm parallel to the oscillation axis at crystal-to-detector distances of 350 mm and 400 mm, respectively. Storage phosphors were scanned with a Kodak image plate scanner, with a pixel size of 0.1 mm in both the x and y directions. The storage phosphor images were analyzed with the program DENZO [from Z. Otwinowski (Department of Molecular Biophysics and Biochemistry of Yale University and Howard Hughes Medical Institute in New Haven, CT)], and indexed reflection intensities were obtained for all the data collected.

Scaling of F_{obs}, calculation of the mean structure factors, statistical analyses, and calculations of the rotation functions were performed by using W. Steigmann's PROTEIN package (31). The final scaling R factor, R_{merg}, was calculated by using the following equation:

$$R_{merg} = \frac{\sum|\langle I\rangle - I|}{\sum|\langle I\rangle|},$$

where I is the intensity of an individual measurement, and $\langle I\rangle$ is the mean intensity of that reflection.

Analytical Methods. Protein in pure enzyme samples was assayed as described by Lowry *et al.* (32). Spectrophotometric measurements and absorbance spectra were recorded on an IBM model 9420 UV–visible spectrophotometer. Electrophoretic characterization was performed as described by Laemmli (33).

RESULTS

Crystallization of $P450_{terp}$. Crystallization trials with $P450_{terp}$ included an analysis of various precipitants, including $(NH_4)_2SO_4$, $MgSO_4$, methylpentanediol, and PEG of average molecular weights 400, 4000, 8000, and 12,000. Only

PEG produced promising results, and initially only showers of microcrystals (mostly of needle or plate-like morphology) could be obtained. Once microcrystals were available, streak seeding was utilized for further screening. A survey of various salts as additives revealed an improved crystal form when divalent cations (especially Mg^{2+}) were present. Thus, the combination of PEG and $MgSO_4$ was found to produce crystals most reliably with a satisfactory three-dimensional shape. Refinement of the concentration and molecular weight of PEG, the concentration of Mg^{2+}, and the pH led to formation of monoclinic crystals from a precipitant solution containing 100 mM Pipes, 16% PEG 8000, 15 mM dithiothreitol, and 40 mM $MgSO_4$ (pH 7.0) at 4°C with dimensions of ≈0.25 × 0.25 × 0.05 mm. Diffraction analysis of these crystals indicated that they diffracted to ≈3.5-Å resolution and belonged to space group $C2$ with approximate unit cell dimensions of $a = 168$ Å, $b = 140$ Å, $c = 114$ Å, and $\beta = 100°$. However, the diffraction intensity was too weak, and the crystals decayed in the x-ray beam too quickly for data collection. In further screening, a hexagonal crystal form was discovered under essentially identical conditions as for the monoclinic crystals, with the exception that PEG 12,000 was used rather than PEG 8000. By adjustment of the pH to 6.4 and use of a ratio of protein-to-precipitant solutions of 3:5 in the original drop mixture, 1.0 × 0.5 × 0.5 mm crystals could be grown (Fig. 1).

Crystallization of the Hemoprotein Domain of $P450_{BM-3}$. Crystals of the hemoprotein domain of $P450_{BM-3}$, grown under the conditions described, were plate-like and grew together as stacks of plates. Streak seeding techniques (28) were helpful in obtaining single crystals, but these crystals were not suitable for high-resolution x-ray diffraction analysis. By lowering the concentration of PEG 8000 in the reservoir to 18% and the protein concentration to 1 mM and by adapting the sitting-drop method (28), large well-formed crystals were obtained (Fig. 2). These crystals are "rocket-shaped" and highly birefringent. Typically, they attain dimensions of 0.8 × 0.5 × 0.2 mm within a day. Use of 10–20% PEG 12,000 in the absence of $MgSO_4$ and dithiothreitol also produced crystals. The morphology and dimensions of these crystals were similar to those obtained with PEG 8000. Crystals of tetragonal morphology were also obtained with 0.8 M $(NH_4)_2SO_4$/10 mM dithiothreithol/0.1 M citrate buffer, pH 6.0, containing 1 mM protein as the final concentration.

SDS/polyacrylamide gel electrophoresis of redissolved crystals of the hemoprotein domain of $P450_{BM-3}$ indicated that the molecular mass of the protein in the crystal was essentially unaltered compared with the purified enzyme. Absorbance spectra were recorded of solutions of redissolved crystals: (*i*) in the absence of added substrate; (*ii*) in the presence of the substrate, 25 μM potassium palmitate; and (*iii*) after sodium dithionite treatment and bubbling with carbon monoxide. These absorbance spectra were comparable to those of the purified, recombinantly produced enzyme in all respects, except for the extent of conversion of the low-spin form to the high-spin form in the presence of an excess of substrate. The cause of this remains to be examined.

Preliminary X-Ray Diffraction Studies. *Analysis of $P450_{terp}$.* The initial diffraction analysis of crystals of $P450_{terp}$ indicated that they belong to the hexagonal space group and diffract to better than 2.8 Å. Precession photographs of the (0*kl*) and (*hk*0) zones were produced utilizing a 0.3-mm collimator, but the extremely long c axis precluded spatial resolution of reflections along that axis. By using a 0.1-mm collimator and taking long (20 hr) precession photographs, it was just possible to resolve spots along the c^* axis in an (0*kl*) zone. Thus, the space group was assigned to be either $P6_1$ or $P6_122$

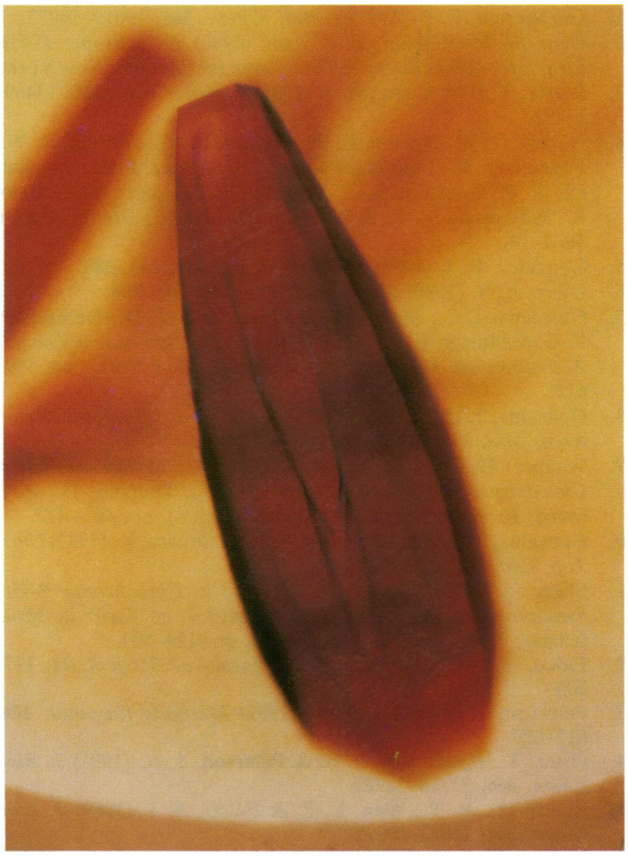

FIG. 1. Crystals of $P450_{terp}$. Dimensions of the crystals are ≈1.0 × 0.5 × 0.5 mm.

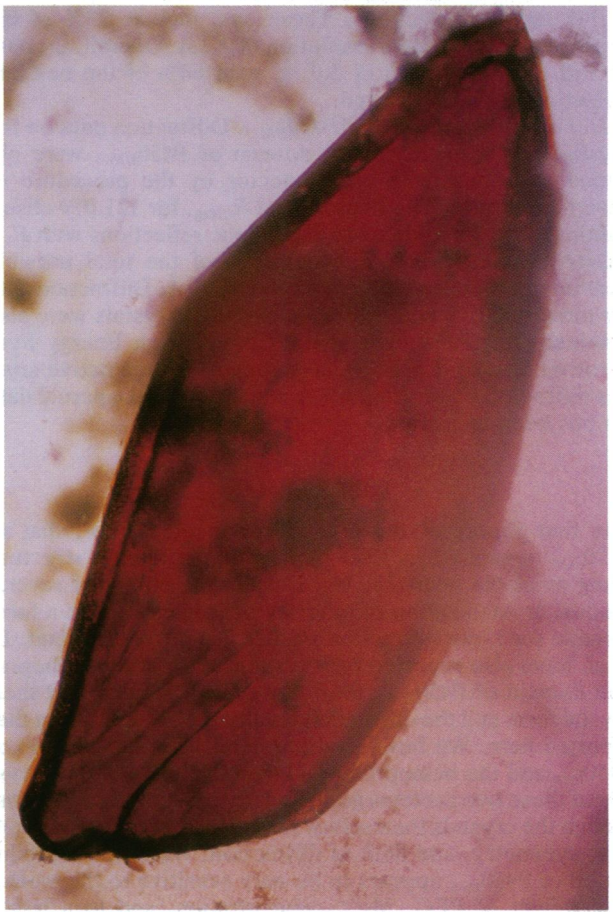

FIG. 2. Crystals of the hemoprotein domain of $P450_{BM-3}$. Dimensions of the crystals are ≈2.0 × 0.6 × 0.35 mm.

with $a = b = 68.9$ Å and $c = 458.7$ Å. To discriminate between the two, an upper level precession photograph was necessary. Because of the long c axis, however, it was unlikely that a truly isolated ($hk0$) or ($hk1$) reciprocal lattice layer could be photographed in the geometry available on our precession camera. Thus, a ($1kl$) zone precession photograph was taken, again with a 0.1-mm collimator, which clearly showed the additional symmetry indicative of space group $P6_122$ or its enantiomorph $P6_522$. Assuming one molecule per asymmetric unit, the calculated V_m (34) is 3.3 Å^3/Da.

Analysis of the hemoprotein domain of P450$_{BM-3}$. Still x-ray photographs of crystals of the hemoprotein domain of P450$_{BM-3}$ showed a measurable diffraction pattern to a resolution limit better than 3.0 Å. Precession photographs of the ($0kl$) and ($hk0$) zones showed that the crystals belong to the space group $P2_1$ with unit-cell dimensions $a = 59.4$ Å, $b = 154.0$ Å, $c = 62.2$ Å, and $\beta = 94.7°$. The crystals were found to remain stable in the x-ray beam for at least 4 days. Calculation of V_m yielded values of 2.6 Å^3/Da if one assumed the presence of a dimer of 110 Da per asymmetric unit or 1.7 Å^3/Da if a trimer were to occupy the same space. Since the value for the trimer lies at the extremes of the distribution of values of V_m found for crystalline proteins (34), we believe that there are two molecules in the asymmetric unit. Subsequently, this was verified by self-rotation calculations using Patterson functions, which indicated the presence of a noncrystallographic twofold axis relating the two monomers in the asymmetric unit.

Diffraction Data. *P450$_{terp}$.* Diffraction data were collected for P450$_{terp}$ at the Cornell High Energy Synchrotron Source. Details of the data collection and subsequent data reduction are provided in *Materials and Methods*. Merging of F_{obs}, with $F_{obs} > 3\sigma(F)$ from three crystals resulted in data with a final scaling R_{merg} of 0.078 for 157,951 observations representing 24,747 unique reflections. These data include 73% of the possible reflections to a resolution of 2.2 Å. Overall, the data extend to a resolution of 2.0 Å, with 60% of the possible reflections being measured.

Hemoprotein domain of P450$_{BM-3}$. Diffraction data on the crystals of the hemoprotein domain of P450$_{BM-3}$ were obtained on a multiwire area detector by the procedure as described in text. The final scaling R_{merg}, for 121,079 observations corresponding to 49,882 unique reflections with $F > 3.0\sigma(F)$, is 0.05. This includes 67% of the total possible number of reflections to a resolution of 2.0 Å. Diffraction data for three putative heavy atom-substituted crystals were also obtained. Recent studies at the Cornell High Energy Synchrotron Source (CHESS) have shown that the native crystals diffract to better than 1.5-Å resolution, and a partial data set has been measured.

DISCUSSION

The first report of the crystallization of a P450, that of P450$_{cam}$, appeared in 1970 (35), with the atomic structure being available later (18, 19). Despite the efforts of several groups, crystallization of other cytochromes P450 in a form suitable for x-ray diffraction studies has proven difficult. Li *et al.* have also reported the crystallization of the hemoprotein domain of P450$_{BM-3}$ (36); however, it is clear that their crystal form and the diffraction quality are different from that reported here. We have reported here the crystallization of P450$_{terp}$ and the hemoprotein domain of P450$_{BM-3}$. The crystals of these two proteins grow under very similar conditions, though the crystals themselves are very different in morphology, symmetry, and limit of diffraction. While the crystallization of P450$_{terp}$ appears to be very sensitive to the molecular weight of PEG, Mg^{2+} concentration, and to a lesser extent pH, crystallization of the hemoprotein domain of P450$_{BM-3}$ is rather insensitive to these parameters. It is also interesting to note that the optimal concentration of protein is very high (compared with usual crystallization reports) in both cases.

The diffraction data collected for these crystals are of good quality. In the case of P450$_{terp}$, the length of the c axis of the unit cell necessitates data collection at a synchrotron facility. For this reason, only native data have been collected. Preliminary molecular replacement calculations for the hemoprotein domain of P450$_{BM-3}$ have been unsuccessful and of P450$_{terp}$ appear to be promising.

The primary goal of the study reported here was to further our understanding of P450 structure–function relationships via the structure determination of representatives of the two classes of P450s. Research in our laboratory has demonstrated that P450$_{terp}$ is a member of class I (mitochondrial) cytochromes P450 based on primary structure and its requirement for an iron–sulfur protein as electron donor for the catalytic oxidation of α-terpineol (39). The utility of P450$_{BM-3}$ as a model for class II (microsomal) P450 systems has been well established in our laboratory (14, 16, 17) and by others (12, 36, 37). Crystallization of these two proteins and the demonstration of their amenability to x-ray diffraction analysis offer promise for the eventual solution of their structures. An analysis of the similarities and differences among these structures and P450$_{cam}$ should provide powerful insights into the function of these complex enzyme systems.

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