J|A|C|S
ARTICLES
Published on Web 04/30/2002

Enhanced Electron Transfer and Lauric Acid Hydroxylation by Site-Directed Mutagenesis of CYP119

Laura S. Koo,[†] Chad E. Immoos,[‡] Michael S. Cohen,[†] Patrick J. Farmer,[‡] and Paul R. Ortiz de Montellano*[,†]

Contribution from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143-0446, and Department of Chemistry, University of California, Irvine, California 92697-2025

Received September 26, 2001

Abstract: CYP119, a cytochrome P450 from a thermophilic organism for which a crystal structure is available, is shown here to hydroxylate lauric acid in a reaction supported by putidaredoxin and putidaredoxin reductase. This fatty acid hydroxylation activity is increased 15-fold by T214V and D77R mutations. The T214V mutation increases the rate by facilitating substrate binding and enhancing the associated spin state change, whereas the D77R mutation improves binding of the heterologous redox partner putidaredoxin to CYP119 and the rate of electron transfer from it to the heme group. A sequence alignment with $P450_{cam}$ can, therefore, be used to identify a part of the binding site for putidaredoxin on an unrelated P450 enzyme. This information can be used to engineer by mutagenesis an improved complementarity of the protein−protein interface that results in improved electron transfer from putidaredoxin to the P450 enzyme. As a result, the catalytic activity of the thermo- and barostable CYP119 has been incorporated into a catalytic system that hydroxylates fatty acids.

Cytochrome P450 enzymes catalyze diverse reactions and accept a wide range of substrates.[1] These reactions include transformations that are very difficult from a synthetic point of view, including the regio- and stereospecific hydroxylation of hydrocarbon chains and the oxidation of halogenated aromatic rings. These properties make cytochrome P450 enzymes highly attractive as potential catalysts in practical processes such as fine chemical synthesis and bioremediation. The enormous practical potential of these enzymes has been limited to date by their instability, the requirement for specific redox partners, the need for expensive and unstable electron donors such as NADH, an incomplete understanding of the determinants of P450 substrate specificity, and a consequent inability to rationally engineer desired specificities and properties into a targeted P450 enzyme.

Efforts to circumvent the limitations on the practical use of P450 enzymes include the development of electrochemical and chemical systems as potential replacements for the NADH or NADPH used in the reactions, although most of these systems still require the presence of a redox partner to mediate the electron transfer to the P450 enzyme.[2−9] Extensive site-specific mutagenesis studies are in progress with CYP101 and CYP102, for which crystal structures are available, to help define the determinants of substrate specificity and to use this information to engineer novel specificities.[10] Although little progress has been made in improving the thermal and other stabilities of P450 enzymes, the recent determination of the crystal structure of CYP119,[11] a thermostable P450 enzyme from *Sulfolobus solfataricus*, provides a model for the development of such enzymes. The thermo- and barostability[12,13] of CYP119 have been attributed to the additive effects of several factors, notable among which are its smaller and more compact structure, an elevated number of ion pairs, and the presence of a "chain" of aromatic residues that spans one side of the molecule.[11,14,15] Experimental work to better define the sources of thermostability in P450 enzymes, particularly those that relate to the unique properties of this hemoprotein, is required if the information is to be employed in the design of novel thermostable P450

* To whom correspondence should be addressed. Fax: (415) 502-4728. E-mail: ortiz@cgl.ucsf.edu.
[†] University of California, San Francisco.
[‡] University of California, Irvine.

(1) Ortiz de Montellano, P. R. In *Cytochrome P450: Structure, Mechanism, and Biochemistry*, 2nd ed.; Ortiz de Montellano, P. R., Ed.; Plenum Press: New York, 1995; pp 473−574.
(2) Estabrook, R. W.; Faulkner, K. M.; Shet, M. S.; Fisher, C. W. *Methods Enzymol.* **1996**, *272*, 44.
(3) Faulkner, K. M.; Shet, M. S.; Fisher, C. W.; Estabrook, R. W. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7705.
(4) Gurev, O. L.; Gilevich, S. N.; Chashchin, V. L. *Biokhimiya* **1990**, *55*, 1160.
(5) Lo, K. K.; Wong, L. L.; Hill, H. A. *FEBS Lett.* **1999**, *451*, 342.
(6) Reipa, V.; Mayhew, M. P.; Vilker, V. L. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 13554.
(7) Remizov, V. A.; Archakov, A. I.; Karyakin, A. V.; Byzova, N. A. *Biokhimiya* **1990**, *55*, 27.
(8) Schwaneberg, U.; Appel, D.; Schmitt, J.; Schmid, R. D. *J. Biotechnol.* **2000**, *84*, 249.
(9) Zhang, Z.; Nassar, A. E. F.; Lu, Z. Q.; Schenkman, J. B.; Rusling, J. F. *J. Chem. Soc., Faraday Trans.* **1997**, *93*, 1769.
(10) Miles, C. S.; Ost, T. W.; Noble, M. A.; Munro, A. W.; Chapman, S. K. *Biochim. Biophys. Acta* **2000**, *1543*, 383.
(11) Yano, J. K.; Koo, L. S.; Schuller, D. J.; Li, H.; Ortiz de Montellano, P. R.; Poulos, T. L. *J. Biol. Chem.* **2000**, *275*, 31086.
(12) Tschirret-Guth, R. A.; Koo, L. S.; Hoa, G. H.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **2001**, *123*, 3412.
(13) McLean, M. A.; Maves, S. A.; Weiss, K. E.; Krepich, S.; Sligan, S. G. *Biochem. Biophys. Res. Commun.* **1998**, *252*, 166.
(14) Chang, Y. T.; Loew, G. *Biochemistry* **2000**, *39*, 2484.
(15) Maves, S. A.; Sligar, S. G. *Protein Sci.* **2001**, *10*, 161.

10.1021/ja017174g CCC: $22.00

enzymes. Furthermore, the evolution of useful catalytic systems employing CYP119 itself is important, as they provide an efficient approach to the development of stable catalytic systems of practical importance.

Neither the natural redox partner(s) of CYP119 in *Sulfolobus solfataricus* nor the endogenous substrate(s) for the enzyme are currently known. In the absence of this information, the catalytic activity of CYP119 has been measured previously using styrene as the substrate in conjunction with putidaredoxin and putidaredoxin reductase, the electron-transfer partners of $P450_{cam}$.[16] As both of these are unnatural complements of CYP119, the catalytic activity that was observed was reproducible but low. To increase this low activity, Thr214, a residue that is not highly conserved in other P450 enzymes but that is adjacent to the highly conserved and catalytically important Thr213, was mutated. Although the crystal structure of the wild-type enzyme indicates that the side chain of Thr214 does not extend into the active site, mutation of Thr214 to an alanine or valine was found in our earlier studies to increase substrate-dependent and -independent dissociation of the water ligand. As dissociation of the water ligand promotes the low- to high-spin shift that enables electron transfer, the mutation was found to increase the catalytic turnover of the enzyme.

Information on the association of P450 enzymes with their redox partners has been obtained mainly from studying the interaction of $P450_{cam}$ with putidaredoxin (Pd) using X-ray crystallography,[17] NMR spectroscopy,[18,19] site-directed mutagenesis,[20−23] chemical modification,[24−27] and other techniques.[28−30] Moreover, the crystal structure of the FMN domain of $P450_{BM-3}$ bound to the heme domain has been determined,[31] and the binding of cytochrome P450 reductase to various mammalian P450 enzymes has also been explored.[32] It is now generally accepted that electrostatic interactions direct and preserve the contact between redox partners, while hydrophobic residues play a key role in anchoring the interaction and facilitating electron flow. Many residues that are important for binding and electron transfer from $P450_{cam}$ to Pd have been identified,[23,33−36] and it has been shown that this interaction can be disrupted by as little as a single point mutation.[20,37] Theoretical and experimental approaches[38−41] have been used to help define the $P450_{cam}$ binding site for Pd, but extrapolation of the proposed binding site information to other P450 enzymes and their partners has not been explored. To our knowledge, extrapolation of this knowledge to the successful engineering of higher affinity partners, particularly if the partners are not closely related, has not been reported.

In the present investigation, we describe site-specific mutagenesis studies that improve the ability of CYP119 to interact with Pd and putidaredoxin reductase (PdR) without causing a loss of enzyme stability or a change in substrate specificity. This enhanced binding interaction in turn increases the catalytic turnover of the P450. We report further that this CYP119 system readily oxidizes fatty acids and that this oxidation can be further improved by mutation of Thr214 in the enzyme active site. This is the first effective catalytic system employing the thermophilic CYP119 enzyme.

Material and Methods

Materials. Styrene, styrene oxide, lauric acid, 12-hydroxylauric acid, trimethylsilyldiazomethane, and all other chemicals were purchased from Aldrich (Milwaukee, WI) and were used without further purification. ^{14}C-labeled lauric acid (55 mCi/mmol) was purchased from American Radio Chemicals (St. Louis, MO). Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was purchased from Regis Technologies (Morton Grove, IL).

General Procedures. UV−vis spectra were recorded on a Cary 1E double beam spectrophotometer. Gas chromatography was performed on an HP5890 Series II chromatograph with a flame ionization detector. A high-pressure liquid chromatography (HPLC) system coupled to a Packard Radiomatic Flow-One model A500 radioisotope detector was used for detection of ^{14}C-labeled compounds. Stopped-flow studies were conducted using a HiTech SF-61DX2 instrument (Hi-Tech Ltd., Salisbury, U.K.).

Construction, Expression, and Purification of CYP119 Mutants. Site-directed mutagenesis of CYP119 was performed as previously described.[16] Briefly, oligonucleotides were obtained from Gibco Life Technologies, Inc. (Grand Island, NY) and used with the Stratagene (La Jolla, CA) QuikChange mutagenesis kit. For the D77R mutant, an *Nru*I site was introduced using the oligo (positions of the mutations are in boldface and new restriction sites are underlined) 5′-GAT CCC CCT CTC CAT **CGC** GAG TTA AGA TCA ATG-3′. An *Nae*I site was introduced into 5′-ATT TTA CTT CTC ATA GC**C** GG**C** AAT GAG ACT **GT**A ACT AAC TTA ATA TC-3′ for T214V. All proteins were expressed and purified as described previously.[16] Yields of the mutants were approximately equal to that of wild-type (20 mg/L of culture) with the exception of the D77R/T214V mutant, which showed an ∼10-fold decrease in yield.

Purification of Pd and PdR. Pd and PdR were overexpressed using clones in pCWori and pET23a, respectively. Pd was expressed and purified according to the methods described by Grayson with the exception that bacterial cells were grown in 2YT.[42] PdR was expressed

(16) Koo, L. S.; Tschirret-Guth, R. A.; Straub, W. E.; Moenne-Loccoz, P.; Loehr, T. M.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **2000**, *275*, 14112.
(17) Raag, R.; Poulos, T. L. *Front. Biotransform.* **1992**, *7*, 1.
(18) Pochapsky, T. C.; Jain, N. U.; Kuti, M.; Lyons, T. A.; Heymont, J. *Biochemistry* **1999**, *38*, 4681.
(19) Mouro, C.; Bondon, A.; Jung, C.; Hoa, G. H. B.; De Certaines, J. D.; Spencer, R. G. S.; Simonneaux, G. *FEBS Lett.* **1999**, *455*, 302.
(20) Unno, M.; Shimada, H.; Toba, Y.; Makino, R.; Ishimura, Y. *J. Biol. Chem.* **1996**, *271*, 17869.
(21) Aoki, M.; Ishimori, K.; Morishima, I.; Wada, Y. *Inorg. Chim. Acta* **1998**, *272*, 80.
(22) Davies, M. D.; Qin, L.; Beck, J. L.; Suslick, K. S.; Koga, H.; Horiuchi, T.; Sligar, S. G. *J. Am. Chem. Soc.* **1990**, *112*, 7396.
(23) Holden, M.; Mayhew, M.; Bunk, D.; Roitberg, A.; Vilker, V. *J. Biol. Chem.* **1997**, *272*, 21720.
(24) Furukawa, Y.; Ishimori, K.; Morishima, I. *Biochemistry* **2000**, *39*, 10996.
(25) Ivanov, Y. D.; Kanaeva, I. P.; Karuzina, I. I.; Archakov, A. I.; Hoa, G. H.; Sligar, S. G. *Arch. Biochem. Biophys.* **2001**, *391*, 255.
(26) Iwuoha, E. I.; Joseph, S.; Zhang, Z.; Smyth, M. R.; Fuhr, U.; Ortiz de Montellano, P. R. *J. Pharm. Biomed. Anal.* **1998**, *17*, 1101.
(27) Wilker, J. J.; Dmochowski, I. J.; Dawson, J. H.; Winkler, J. R.; Gray, H. B. *Angew. Chem., Int. Ed.* **1999**, *38*, 90.
(28) Aoki, M.; Ishimori, K.; Fukada, H.; Takahashi, K.; Morishima, I. *Biochim. Biophys. Acta* **1998**, *1384*, 180.
(29) Shimada, H.; Nagano, S.; Hori, H.; Ishimura, Y. *J. Inorg. Biochem.* **2001**, *83*, 255.
(30) Unno, M.; Christian, J. F.; Benson, D. E.; Gerber, N. C.; Sligar, S. G.; Champion, P. M. *J. Am. Chem. Soc.* **1997**, *119*, 6614.
(31) Sevrioukova, I. F.; Li, H. Y.; Zhang, H.; Peterson, J. A.; Poulos, T. L. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 1863.
(32) Cvrk, T.; Strobel, H. W. *Arch. Biochem. Biophys.* **2001**, *385*, 290 and references therein.
(33) Nakamura, K.; Horiuchi, T.; Yasukochi, T.; Sekimizu, K.; Hara, T.; Sagara, Y. *Biochim. Biophys. Acta* **1994**, *1207*, 40.
(34) Stayton, P. S.; Sligar, S. G. *Biochemistry* **1990**, *29*, 7381.
(35) Shimizu, T. *Recent Res. Dev. Pure Appl. Chem.* **1997**, *1*, 169.
(36) Yasukochi, T.; Okada, O.; Hara, T.; Sagara, Y.; Sekimizu, K.; Horiuchi, T. *Biochim. Biophys. Acta* **1994**, *1204*, 84.
(37) Koga, H.; Sagara, Y.; Yaoi, T.; Tsujimura, M.; Nakamura, K.; Sekimizu, K.; Makino, R.; Shimada, H.; Ishimura, Y.; Yura, K.; Go, M.; Ikeguchi, M.; Horiuchi, T. *FEBS Lett.* **1993**, *331*, 109.
(38) Pochapsky, T. C.; Lyons, T. A.; Kazanis, S.; Arakaki, T.; Ratnaswamy, G. *Biochimie* **1996**, *78*, 723.
(39) Roitberg, A. E. *Biophys. J.* **1997**, *73*, 2138.
(40) Roitberg, A. E.; Holden, M. J.; Mayhew, M. P.; Kurnikov, I. V.; Beratan, D. N.; Vilker, V. L. *J. Am. Chem. Soc.* **1998**, *120*, 8927.
(41) Stayton, P. S.; Poulos, T. L.; Sligar, S. G. *Biochemistry* **1989**, *28*, 8201.

by inoculating 2 L of 2YT medium with 25 mL of an overnight culture grown from a single colony at 30 °C in 2YT medium containing ampicillin. Protein expression in *E. coli* BL21(DE3) cells was induced using 1 mM IPTG after the cell density reached an o.d. of 0.6 at 30 °C. Cells were shaken for another 3 h and then harvested and lysed using lysozyme in 50 mM Tris·Cl buffer, pH 7.4 at 4 °C. After sonication and centrifugation, the supernatant was subject to 30% and 70% ammonium sulfate cuts. After dialysis against 20 mM potassium phosphate buffer (KP_i), pH 7.4, the protein solution was loaded onto a Q Sepharose column (30 cm × 2.5 cm), washed with 20 mM KP_i, pH 7.4, buffer with 100 mM KCl, and then eluted using a gradient of 100−400 mM NaCl. The yellow fractions were combined, ammonium sulfate was added to 30%, and the solution was loaded onto a Phenyl Sepharose column (5 cm × 2.5 cm). Protein was eluted using a gradient of 30−0% ammonium sulfate in 20 mM KP_i buffer and dialyzed overnight against 50 mM KP_i, pH 7.4, 100 mM KCl, and 5% glycerol.

Characterization of Mutants. The shift of the enzyme's Soret absorbance maximum from 415 to 390 nm indirectly measures the shift from the low- to the high-spin states of cytochrome P450 that correlates with the loss of the distal water ligand from the iron atom. The UV−vis spectra of the substrate-free enzymes were recorded, and the A415/A390 ratio was compared to that for wild-type CYP119. CO-difference spectra were obtained to confirm the presence of the thiolate ligand to the heme iron. Briefly, excess dithionite was added to 5−10 μm enzyme in a cuvette, and CO was bubbled into the solution. $\Delta A450 - \Delta A490$ was calculated after subtraction of the dithionite reduced spectrum from the CO-bound spectrum, with $\epsilon_{450} = 100\ mM^{-1}$. All T_m measurements were performed on a Jasco J-715 CD spectrophotometer fitted with a Peltier water bath. A 5 μM enzyme concentration in 50 mM potassium phosphate buffer, pH 7.0, containing 100 mM KCl was used. The melting point for wild-type CYP119 and each of its mutants was determined by monitoring the change in molar ellipticity at 221 nm over the temperature range from 40 to 100 °C.

Stopped-Flow Measurements and Data Analysis. Anaerobiosis of the instrument was achieved by incubating the stopped-flow apparatus in 50 mM bis-Tris buffer with glucose/glucose oxidase overnight. Anaerobic samples were prepared by five cycles of alternately evacuating and purging with oxygen-scrubbed argon in a tonometer. High purity carbon monoxide was then anaerobically bubbled through the solution before transfer to the stopped-flow syringe. All the reactions were conducted at 37 °C. The final concentrations of the assay components were 500 nM CYP119, 10 μM Pd, 500 nM PdR, 360 μM lauric acid, 10 mM glucose, 0.05 mg/mL of glucose oxidase, 0.1 mg/mL of SOD, 0.1 mg/mL of catalase, 250 μM NADH, and CO in 50 mM bis-Tris, pH 7.0. Single beam mode photodiode array data of "single turnover" electron transfer were collected. Formation of the one-electron reduced P450-CO complex at 450 nm was plotted against time using Kaleidagraph (Synergy Software, Reading, PA) and fit to a first-order exponential equation.

Electrochemical Methods. A BAS 100 W electrochemical analyzer was used with a standard three-electrode configuration containing a Pt wire counter electrode, a Ag/AgCl reference electrode (BAS), and a basal-plane pyrolytic graphite (PG) disk working electrode. All electrochemical experiments were performed at room temperature (22 ± 2 °C) in 50 mM potassium phosphate buffer solution, pH 7.0, containing 0.1 M NaBr as supporting electrolyte. Film-modified electrodes were prepared by depositing 10 μL of 0.01 M aqueous didodecyldimethylammonium bromide (ddab) and 10 μL of 0.3 mM protein onto a graphite disk electrode. The protein/didodecyldimethylammonium bromide films were dried in a closed vessel overnight and then exposed to air for at least 24 h or until completely dried prior to electrochemical experiments. To remove oxygen prior to experiments, solutions were purged with nitrogen for at least 20 min, and a nitrogen atmosphere was maintained over the solutions at all times.

Binding Studies and Activity Assays. Lauric acid binding constants (K_s) were determined at 40 °C by difference spectroscopy. Lauric acid in a 50/50 solution of DMF/CH_3CN was titrated to less than 1% of the initial volume into the sample cuvette containing 1.33 μM CYP119 in 50 mM bis-Tris buffer, pH 6.0 or buffer containing 500 μM imidazole. Equal amounts of DMF/CH_3CN were titrated into the reference cuvette, and the shift from 416 to 390 nm was followed. In the case of Pd binding to CYP119, Pd in 50 mM bis-Tris buffer, pH 7.0 was titrated into a sample cuvette of 5 μM CYP119 and a reference cuvette containing buffer only. The shift from 413 to 440 nm was followed. K_s was then determined by fitting plots of $\Delta A_{390} - A_{416}$ or $\Delta A_{440} - A_{413}$ against substrate concentration[43] to the quadratic equation:

$$\Delta A = \frac{A_{max}}{[E]}\left(\frac{K_s + [E] + [L] - \sqrt{(K_s + [E] + [L])^2 - 4[E][L]}}{2}\right)$$

Styrene epoxidation was measured with 1 μM CYP119, 20 μM Pd, 1 μM PdR, 7.5 mM styrene, 1 mg/mL of catalase, and 6 mM NADH in 50 mM bis-Tris buffer, pH 7.0 at 37 °C. Styrene epoxide was quantitated by gas−liquid chromatography (GC) as described previously using ethylbenzene as the internal standard.[16] The rate of lauric acid hydroxylation was determined using 500 nM CYP119, 10 μM Pd, 500 nM PdR, excess catalase, and 3 mM NADH in 50 mM bis-Tris buffer, pH 7.0 and 10−360 μM 1:5 ^{14}C-labeled lauric acid. The reaction mixture was incubated at 37 °C and was then quenched with 6% sulfuric acid in acetonitrile. The samples were injected directly onto the column after centrifugation to remove any precipitate that might be present. The reaction products were separated on an Alltech Econosil C18 column (3.2 × 250 mm) connected to a radiomonitor. An isocratic elution profile of 90:10:0.1 acetonitrile:water:trifluoroacetic acid gave retention times of 3.4 min for all hydroxylauric acid isomers and 5.2 min for lauric acid.

GC-MS Identification of Reaction Products. Scaled-up samples of lauric acid with CYP119, Pd, and PdR were prepared. After the reaction was completed, samples were acidified with HCl and extracted with diethyl ether. The ether extracts were combined, dried over anhydrous sodium sulfate, and concentrated to dryness on a rotary evaporator. Derivatization for GC-MS analysis was carried out as previously described.[44] Methylation of the product was carried out in 80:20 benzene:MeOH using trimethylsilyldiazomethane as described by Hashimoto et al.[45] The methylations were quenched with acetic acid. After evaporation of the solvent, 2 μL of pyridine and 18 μL of BSTFA with 1% TMCS were added to the sample. The reaction was allowed to proceed at 60 °C for 1 h; GC separation was obtained on a DB-1 column (10 m × 0.25 i.d.) heated from 100 to 150 °C at 20 °C/min and then 4 °C/min to 250 °C. The column was coupled to a VG70S double focusing mass spectrometer in EI sample ionization mode (70 eV).

Results

Construction and Characterization of the D77R Mutants. In an effort to adapt CYP119 for use with Pd and PdR, the electron donor partners for $P450_{cam}$, CYP119 was aligned with $P450_{cam}$, and potential nonideal protein−protein interactions within the putative reductase docking site were identified. In the docking of $P450_{cam}$ to Pd, several key arginine residues within the proposed $P450_{cam}$ binding site are believed to associate with aspartate residues on the Pd site. Some representative interactions between the two proteins, where the first residue in each pair is on $P450_{cam}$ and the second on Pd, are

(42) Grayson, D. A.; Tewari, Y. B.; Mayhew, M. P.; Vilker, V. L.; Goldberg, R. N. *Arch. Biochem. Biophys.* **1996**, *332*, 239.
(43) De Rosa, M.; De Rosa, S.; Gambacorta, A.; Minale, L.; Thompson, R. H.; Worthington, R. D. *J. Chem. Soc., Perkin Trans. 1* **1977**, *6*, 653.
(44) Buhler, D. R.; Miranda, C. L.; Deinzer, M. L.; Griffin, D. A.; Henderson, M. C. *Drug Metab. Dispos.* **1997**, *25*, 1176.
(45) Hashimoto, N.; Aoyama, T.; Shioiri, T. *Chem. Pharm. Bull.* **1981**, *29*, 1475.

Arg72−Asp38, Arg109−Asp34, Arg112−Asp38/103, and Arg364−Asp9. On the basis of our sequence alignment, the corresponding pairs on CYP119 and Pd if binding is in the same region of the protein would be Lys30−Asp38, Asp77−Asp34, Arg80−Asp38/103, and Arg324−Asp9:

CYP119 71SDPPLH**D**ELRSMS83

$P450_{cam}$ 103MDPPEQ**R**QFRALA115

Three of four CYP119−Pd interactions parallel the $P450_{cam}$−Pd ones, but the Asp77−Asp34 interaction would be expected to be highly unfavorable due to charge repulsion and would hinder the formation of a productive CYP119−Pd complex. Calculation of the CYP119 electrostatic potential as compared to that of $P450_{cam}$ supports this hypothesis as D77 was found to be located in a highly electronegative patch (Figure 1a), whereas R109 of $P450_{cam}$ helps to form an electropositive region (Figure 1b). Mutation of Asp77 into an arginine would be expected to alleviate the charge repulsion if the binding site has been correctly identified (Figure 1c). In addition to this mutant, the D77R mutation was also introduced into the T214V mutant, as the Thr214 mutation improves the binding of substrates and increases the proportion of the high-spin state present in the protein.

The three mutants are indistinguishable by CO-difference spectroscopy and circular dichroism from wild-type CYP119. In addition, the D77R mutation did not alter the high- to low-spin ratio of the substrate free form as compared to the wild-type or the T214V protein. The melting points as determined by CD spectroscopy of the mutant proteins (88−92 °C) are comparable to that of the wild-type enzyme (91 °C).[16] On the basis of its interaction with the PBE94 column and its behavior in the isoelectric focusing gel, the pI values of the D77R mutants are higher than those of wild-type CYP119 and its T214V mutant (Figure 2). In addition, extra bands are present in the IEF gel that are not due to impurities of the CYP119 preparation. These bands may be related to the finding of two interconverting forms of the protein when it is eluted from the purification columns.

First Electron Transfer from Pd to CYP119. Stopped-flow experiments were used to measure the rate of the first electron transfer between putidaredoxin and CYP119 at 37 °C under anaerobic conditions. The final assay system included a 1:20:1 ratio of CYP119:Pd:PdR, a glucose/glucose oxidase oxygen scavenging system, lauric acid, catalase, superoxide dismutase, CO, and NADH in 50 mM bis-Tris, pH 7.0. As described later, lauric acid was investigated as a substrate after a range of other small molecules, including styrene, was found to be poor substrates.

The sequential set of steps required for electron transfer to a P450 from its electron-transfer partner can be described by a first-order exponential equation. This hypothesis, which is based on the assumption that the rate of the actual electron transfer is much slower than those of protein association and CO binding, is consistent with previous experiments on $P450_{cam}$.[46−49] The rates obtained with wild-type CYP119 and the D77R, T214V, and D77R/T214V mutants are shown in Table 1. The D77R mutation clearly enhances electron transfer, giving rise to rates that are 5 times those of the corresponding wild-type and T214V parent enzymes. The slight increases in electron transfer in the T214V mutants over the parent wild-type and D77R enzymes correlate with the slight decreases in the spectroscopically determined binding constant (K_s) for lauric acid (see below).

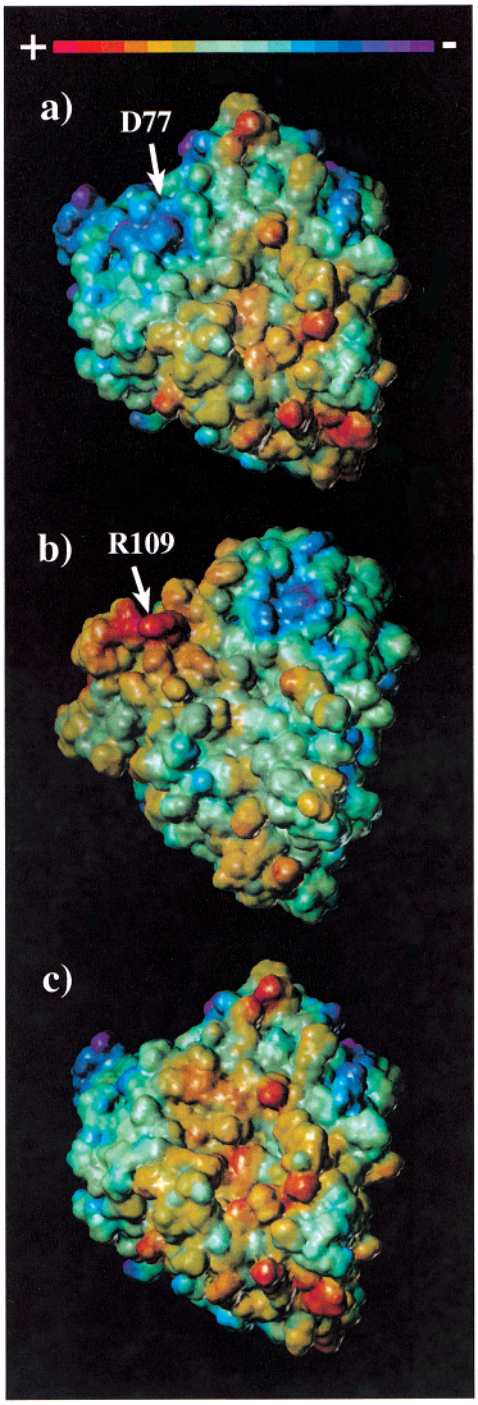

Figure 1. Electrostatic potential mapped onto the molecular surface of the proposed Pd binding region of CYP119 and $P450_{cam}$: (a) wild-type CYP119; (b) $P450_{cam}$; and (c) D77R CYP119. Surface potentials were calculated using formal charges in SYBYL×aeÆ 6.7.1 (Tripos Inc., 1699 South Hanley Rd., St. Louis, Missouri, 63144) and mapped onto the protein surfaces using the MOLCAD module of SYBYL. Measured redox potentials are reported as ±5 mV.

(46) Brewer, C. B.; Peterson, J. A. *J. Biol. Chem.* **1988**, *263*, 791.
(47) Brewer, C. B.; Peterson, J. A. *Arch. Biochem. Biophys.* **1986**, *249*, 515.
(48) Hintz, M. J.; Mock, D. M.; Peterson, L. L.; Tuttle, K.; Peterson, J. A. *J. Biol. Chem.* **1982**, *257*, 14324.
(49) Hintz, M. J.; Peterson, J. A. *J. Biol. Chem.* **1981**, *256*, 6721.

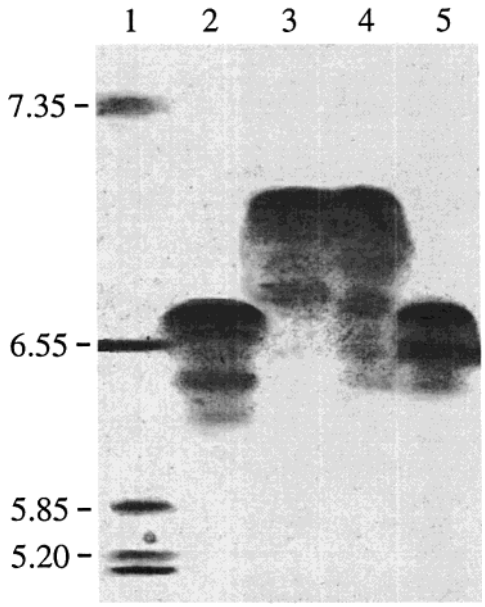

Figure 2. IEF gel of CYP119 and D77R. Lane 1, Pharmacia broad IEF markers; lane 2, 10 μg of wt-CYP119; lane 3, 10 μg of D77R CYP119; lane 4, 10 μg of D77R/T214V CYP119; lane 5, 10 μg of T214V CYP119.

Table 1. Effect of Mutations on CYP119 Electron Transfer and Catalysis[a]

enzyme	first electron-transfer rate (min^{-1})	lauric acid hydroxylation k_{cat} (min^{-1})	Pd binding K_d (mM)
CYP119, wild-type	0.65 ± 0.03	0.36 ± 0.01	2.1 ± 0.7
T214V	0.90 ± 0.04	2.08 ± 0.01	
D77R	3.30 ± 0.20	4.71 ± 0.03	0.5 ± 0.3
D77R/T214V	4.47 ± 0.06	8.80 ± 0.30	

[a] Values are reported as the mean of triplicate determinations $\pm$ SD.

Table 2. Midpoint Potential (E_m) of CYP119 versus an Ag/AgCl Electrode

enzyme	redox potential (mV)
CYP119, wild-type	-214 ± 5
CYP119, D77R	-227
$P450_{cam}$	-216

Putidaredoxin Binding by CYP119. Addition of Pd to CYP119 gives a spectral change analogous to that observed with $P450_{cam}$. Difference spectroscopy reveals a trough at 413 nm and a peak at 440 nm. The K_s value for lauric acid-bound CYP119 is 2.1 mM (Table 1). The D77R mutation decreases this value to 500 μM. Literature values for the binding of Pd to camphor-bound $P450_{cam}$ range between 5 and 17 μM.[21,22,37,50]

Redox Potential of the D77R Mutants. The redox potentials of wild-type CYP119 and the D77R mutant were measured to determine whether the D77R mutation had any effect on the redox potential of the protein, as a change in redox potential could also lead to an increase in electron-transfer rate (Table 2). There is no significant change in the potential of the D77R mutant as compared to that of the wild-type protein.

Substrate Binding by CYP119. Previous studies of the catalytic turnover of CYP119 employed styrene as the substrate even though styrene is turned over very poorly by the enzyme. A further search for enzyme ligands identified fatty acids as potential substrates. CYP119 is able to bind C8−C20 saturated fatty acids with varying degrees of affinity, as judged from the magnitude of the spin state change and the magnitude of the linked absorbance shift (Figure 3). K_s values were determined for capric (C10) through arachidic (C20) acid (Table 3).

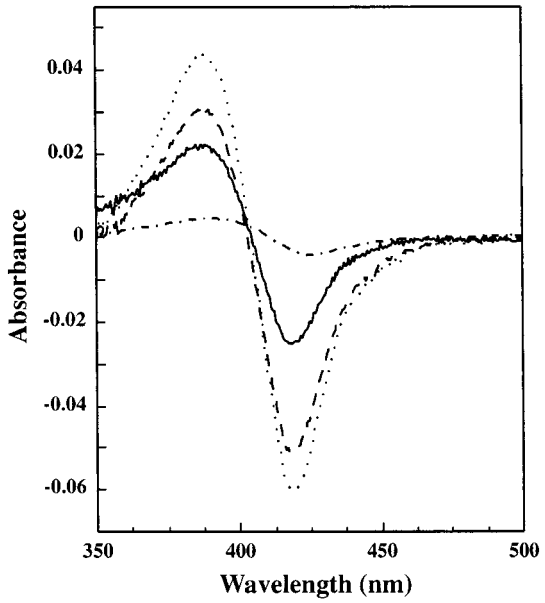

Figure 3. UV−vis difference spectra of CYP119 bound to saturated fatty acids. Overlay of 1.3 μM CYP119 bound to saturating amounts of C10 (− − −), C12 (· · ·), C20 (−) saturated fatty acids, and styrene (− · − · −).

Table 3. Substrate Binding to Wild-type CYP119

substrate	K_s (μM)
capric acid (C10)	28 ± 2
lauric acid (C12)	1.2 ± 0.2
myristic acid (C14)	1.0 ± 0.2
palmitic acid (C16)	0.7 ± 0.2
stearic acid (C18)	1.3 ± 0.2
arachidic acid (C20)	5 ± 1

All four CYP119 proteins studied here bound lauric acid much more tightly than styrene, for which the K_s value for binding to the wild-type enzyme was 530 μM. However, the CYP119 mutants had different fatty acid binding properties relative to the wild-type protein (Table 4). The binding constants of lauric acid to wild-type CYP119 and its D77R mutant are very similar, as might be expected due to the distance between the mutation and the distal (substrate-binding) side of the active site (Figure 4). On the other hand, the K_s values for the T214V and D77R/T214V mutants show a 5-fold decrease and correlate well with the presence of a higher proportion of the high-spin protein in their spectra. Lauric acid was selected for the enzyme assays because of its higher solubility, the availability of standards for its metabolites, and the extensive literature relevant to identification of its metabolites.

Fatty Acid Hydroxylation. CYP119 catalyzes the oxidation of lauric acid at 37 °C using Pd, PdR, and NADH with an optimized enzyme ratio of 1:20:1 CYP119:Pd:PdR. Catalase was included in all the incubations to eliminate any H_2O_2 that might be produced by uncoupling and thus to prevent H_2O_2-supported reactions. Under these conditions, all the mutants exhibited an increase in activity with respect to the wild-type enzyme (Table 1). Whereas wild-type CYP119 turns over lauric acid at a rate

(50) Lipscomb, J. D. *Biochemistry* **1980**, *19*, 3590.

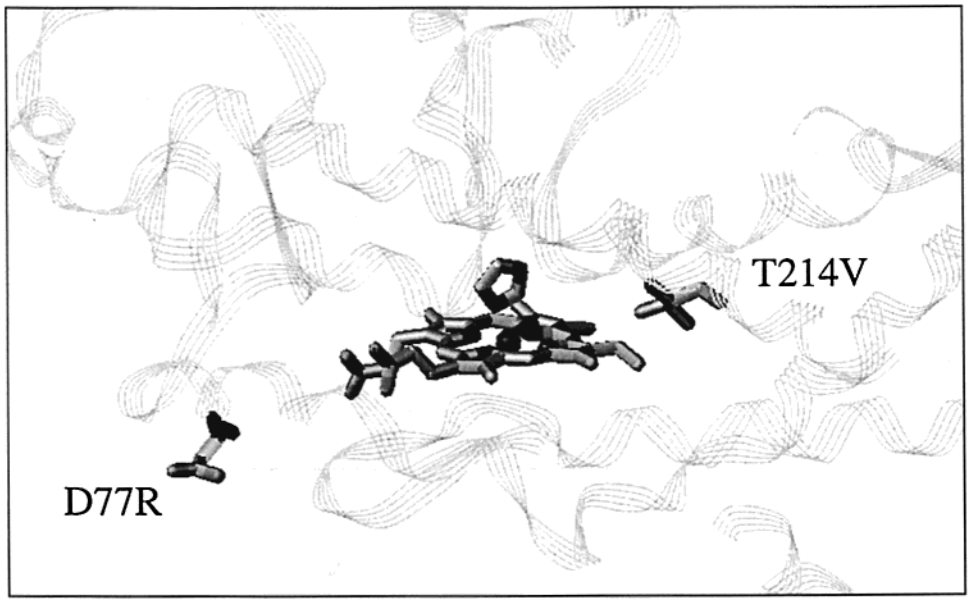

Figure 4. Cutaway of the CYP119 crystal structure showing the position of the D77 and T214 residues relative to the heme moiety.

Table 4. Effect of T214V Mutation on CYP119 Substrate Binding[a]

enzyme	lauric acid K_s (μM)	lauric acid K_M (μM)	styrene epoxidation V_{max} (min^{-1})	styrene K_s (μM)
CYP119, wild-type	1.4 ± 0.2	21 ± 1	0.05 ± 0.01	530 ± 90
D77R	1.5 ± 0.1	30 ± 1	0.07 ± 0.04	
T214V	0.4 ± 0.1	11 ± 1	0.20 ± 0.10	120 ± 10
D77R/T214V	0.3 ± 0.1	10 ± 3	0.20 ± 0.01	

[a] Values are reported as the mean of triplicate determinations ± SD. Styrene oxidation assays contained 1 μM CYP119, 20 μM Pd, 1 μM PdR, 7.5 mM styrene, 1 mg/mL of catalase, and 6 mM NADH in 50 mM bis-Tris buffer, pH 7.0 at 37 °C.

Table 5. Regiospecificity of Lauric Acid Hydroxylation by CYP119 and Its Mutants

enzyme	ω-1 %	ω-2 %	ω %	ω-3 %
wild-type CYP119	70	22	7	1
D77R	69	21	8	2
T214V	76	16	6	2
D77R/T214V	70	16	10	4

of only 0.36 min^{-1}, the D77R mutant hydroxylates lauric acid at a rate of 4.71 min^{-1}. The T214V mutation alone increases the rate to 2.08 min^{-1}, but the double D77R/T214V mutation raises it even further to 8.80 min^{-1}.

Identification of Lauric Acid Hydroxylation Product(s). The metabolites of lauric acid formed by wild-type CYP119 and its mutants were identified using tandem GC-MS. The fragmentation patterns of the methylated and trimethylsilylated hydroxylauric acid isomers gave the expected peaks:[51−53] 12-hydroxylauric acid (*m*/*z* 89, 103, 255, and 287), 11-hydroxylauric acid (*m*/*z* 117, 185, and 287), 10-hydroxylauric acid (*m*/*z* 131, 273, and 287), and 9-hydroxylauric acid (*m*/*z* 145, 259, and 287). Direct comparison of the experimental samples with standards of the ω- and ω-1 hydroxyl derivatives and the reported fragmentation patterns show that CYP119 and its mutants hydroxylate lauric acid at the ω- through ω-3 positions. The regiospecificity is unchanged in the mutants and wild-type protein, even for the T214V mutants, and gives predominantly the ω-1 product (~70%) followed by a smaller amount of the ω-2 (~20%) (Table 5).

Styrene Epoxidation. When styrene epoxidation by the T214V mutants was compared using Pd, PdR, and NADH as the electron donor system, the catalytic activity, albeit still low, was significantly increased over that of wild-type CYP119 (Table 4). Interestingly, the D77R mutation does not affect the styrene epoxidation rate, but the T214V mutant enhances the rate 3- to 4-fold. Wild-type CYP119 showed a V_{max} of 0.05 min^{-1}, while the T214V mutant gave a V_{max} of 0.20 min^{-1}. A similar increase was seen between the D77R mutant and the D77R/T214V double mutant. These results suggest that, in the case of styrene, substrate binding and the spin state change are the limiting factors rather than electron transfer.

Discussion

Previous efforts to support CYP119 catalytic turnover with alternate electron donor proteins have included the use of Pd and PdR, the natural redox partners of P450$_{cam}$. It was evident from these initial experiments with Pd/PdR that increased efficiency of the system would be desirable. On the basis of the hypothesis that the corresponding region of CYP119 most likely complements the surface of Pd in a different manner than the binding region of its native but not yet identified partner, the sequence alignment of CYP119 with P450$_{cam}$ was examined to locate regions suitable for site-directed mutagenesis. Because of the high structural identity between these two P450s, the sequence alignment did reveal one potentially repulsive interaction between CYP119 and Pd. Assuming, of course, a similar docking mode, the Arg109−Asp34 ion pair between P450$_{cam}$ and Pd was predicted to be replaced in the CYP119−Pd interaction by an Asp77−Asp34 repulsive interaction.

To create a tighter CYP119−Pd interface, Asp77 of CYP119 was mutated to an arginine. By determining the rate of lauric acid turnover, the rate of first electron transfer, and several

(51) Eglinton, G.; Hunneman, D. H.; McCormick, A. *Org. Mass Spectrom.* **1968**, *1*, 593.
(52) Lemaire, P.; Lafaurie, M.; Weissbart, D.; Durst, F.; Pflieger, P.; Mioskowski, C.; Salaun, J. P. *Lipids* **1992**, *27*, 187.
(53) Shoun, H.; Sudo, Y.; Beppu, T. *J. Biochem.* **1985**, *97*, 755.

spectral and physical constants for the mutants incorporating D77R, evidence was obtained to demonstrate that the improved catalytic activity of the mutant proteins is, in fact, a direct result of the mutation. We conclude, therefore, that it is possible to optimize the interface between a cytochrome P450 and a redox partner by mutations at proposed contact points and that the resulting catalytic improvement can be attributed to simple electrostatic interactions between amino acid residues.

Two mutants were constructed incorporating the D77R change, the single mutant, and a D77R/T214V double mutant that incorporates a mutation previously shown to increase the proportion of the protein in the high-spin (reducible) state.[16] As suggested previously by both the crystal structure of CYP119 and the active site topology studies,[54] Thr214 makes no direct contact with the active site itself even though the T214V mutant exhibits enhanced substrate binding. Combining the D77R and T214 mutations thus results in a higher rate of catalysis due to the combination of increased substrate binding and high-spin state with an increased rate of reduction of the protein by Pd.

The basic properties of the parent enzyme were unaffected by introduction of the D77R mutation whether it was in the wild-type protein or the T214V single mutant. The relative ratios of the low- and high-spin states of the substrate free form remained unchanged, the protein formed spectroscopically indistinguishable reduced-CO complexes, and the enzymes had similar melting temperatures (T_m's). Because Asp77 is located on the surface of the protein on the proximal rather than substrate-binding distal side (Figure 4), the mutation also was not expected to alter the affinity of the enzyme for lauric acid, the fatty acid substrate (Table 3). In accord with this, the K_s of each D77R mutant for the binding of lauric acid remained the same within error relative to the appropriate parent protein (1.4 and 1.5 μM for wild-type and D77R, 0.42 and 0.32 μM for T214V and D77R/T214V, respectively). The only physical change detected was a slightly higher pI value for the D77R proteins based on their isoelectric focusing gel properties (Figure 2), but this was expected for replacement of a negatively charged, solvent exposed surface residue by a positively charged one.

The mutant proteins were expected to have very different surface electrostatics (Figure 1) than wild-type CYP119, and, indeed, the D77R mutant was reduced 5 times faster by Pd than the parent protein (Table 1). The wild-type and T214V mutants had first electron-transfer rates that nearly agreed (0.65 and 0.90 min^{-1}) under saturating lauric acid conditions. Introduction of the D77R mutation into both of these proteins improved the rates for their first electron transfer 5-fold to 3.3 and 4.47 min^{-1}, respectively.

The increased rate of first electron transfer was matched by a greater than 10-fold increase in the rate of lauric acid hydroxylation (Table 1). The D77R mutation alone increased Pd binding 4-fold and the rate of lauric acid oxidation 13-fold. The increase in rate for the T214V mutation over wild-type enzyme can be attributed to the decrease in K_s for lauric acid and the increase in the high-spin fraction of the enzyme and the double D77R/T214V mutant showed an activity equal to the product of the two individual increases. This cooperativity of the mutations is consistent with the proposed role for the mutation since for optimal turnover, a P450 should be both 100% high spin with substrate bound and should be complemented by its natural reductase partners. A compromise in one or the other of these parameters results in a decrease in the catalytic activity. It follows then that improving the CYP119−Pd interface and the proportion of the protein in the high-spin state will lead to higher turnover numbers.

The measured steady state turnover rates are twice those of the measured electron-transfer rate. The origin of this difference is unclear but presumably stems from an unidentified but systematic error in the methodology employed to measure the electron-transfer rate. However, the difference is not relevant to the conclusions of this study as it is similar for both the wild-type and the mutant proteins.

The evidence is consistent with the proposal that the D77R mutation plays a largely electrostatic role in enhancing the association of CYP119 with Pd. The increase in electron transfer and in substrate hydroxylation is consistent with the design principle that the D77R mutant would eliminate a repulsive interaction in the binding of Pd to CYP119. In turn, these findings also suggest that both the hypothesis of a conserved docking site for Pd on CYP119 and the protein alignment used to select the interacting residues were essentially correct. The dissociation constants for the binding of Pd to CYP119 further support these conclusions and provide direct evidence that the D77R mutant is bound with higher affinity.

Addition of Pd to CYP119 gives a spectral change (data not shown) similar to the one reported for Pd and $P450_{cam}$. A comparison of the rates of electron transfer from Pd to $P450_{cam}$ and CYP119 suggests that the association constant for the binding of Pd to CYP119 is much higher than that for binding to $P450_{cam}$. This is, in fact, true since the K_s for Pd and CYP119 was determined to be 2.1 mM (Table 1), 2 orders of magnitude greater than for $P450_{cam}$. The D77R mutation reduced this number by more than 4-fold, which correlates well with the 5-fold increase in the electron-transfer rate observed for this protein over wild-type CYP119.

The final experiment performed to localize the observed effects to the D77R mutation alone was to determine the redox potential of CYP119. If the differences between the mutants and wild-type CYP119 are due to a difference in the electrostatic interaction of Pd with CYP119, then the redox potential of the P450 should be unaffected by the D77R mutation. If, however, the mutation had an unpredicted effect transparent to the spectroscopic methods used to characterize the protein such as subtle global changes in structure, the redox potential would almost surely be grossly affected. Using the surfactant film method, a slight difference in redox potential was observed (13 mV) (Table 2), but this difference is very close to the error of the method. It is, therefore, evident that the redox potential of CYP119 is essentially unchanged upon the introduction of the D77R mutation.

The natural substrate of CYP119 is not known, although one possibility is that it is a sulfur compound as *Sulfolobus solfataricus* grows lithotrophically in sulfur-, sulfide-, or tetrathionate-rich media. Efforts to identify surrogate substrates by scanning a library of small organic compounds led first to the identification of styrene as a poor substrate that could only be turned over with H_2O_2 rather than a surrogate electron-transfer system. As reported here, however, the enzyme appears

(54) Tschirret-Guth, R. A.; Koo, L. S.; Ortiz de Montellano, P. R. *J. Biol. Inorg. Chem.* **2000**, *5*, 204.

to effectively bind (Figure 3) and hydroxylate fatty acids of various chain lengths using Pd and PdR as surrogate electron-transfer proteins. The predominant reaction with lauric acid is ω-1 hydroxylation (Table 5). Previous studies have shown that ω-1 hydroxylation is thermodynamically favored and is predominant in P450 systems that do not have specific mechanisms determining the site of oxidation.[55] Although fatty acids are suitable as experimental substrates, it is unlikely that they are the natural substrates as no fatty acids have been detected in *Sulfolobus*.[56] Further, the lipids of *Sulfolobus solfataricus* are generally ether lipids that are devoid of ester linkages.[57,58]

Styrene was also examined as a substrate for the CYP119 mutants as its epoxidation by CYP119 and H_2O_2 was previously investigated.[16] Surprisingly, styrene behaved differently from lauric acid in that the epoxidation rate was not increased by the D77R mutation. This contradiction can be explained, however, by the large difference in binding affinity of CYP119 for the two substrates. Lauric acid gives an easily measured spectroscopic binding constant, whereas the binding of styrene was only firmly established by the use of NMR techniques.[16] The T214V mutation thus increases the rate of oxidation of styrene because it improves the ability of styrene to bind and cause the required low to high-spin shift, but the D77R mutation has no effect because the weak interaction with styrene makes substrate binding and the spin state shift rate-limiting rather than the electron transfer that is accelerated by the D77R mutation.

The present findings demonstrate that the binding site for Pd on a new and unrelated P450 enzyme can be inferred from a sequence comparison with $P450_{cam}$ and that this information can be used to construct a functional hydroxylation system employing a surrogate electron donor protein. The construction of such systems is important if a library of P450 enzymes is to be developed for use in practical applications such as fine chemical synthesis. Work is continuing in this laboratory to identify the natural reductase partners for the enzyme, as their isolation would make possible the construction of a high-temperature hydroxylation system.

Acknowledgment. This work was supported by National Institutes of Health Grant GM25515. We thank Felice Lu for helpful suggestions and assistance with SYBYL.

JA017174G

(55) CaJacob, C. A.; Chan, W. K.; Shephard, E.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1988**, *263*, 18640.
(56) Langworthy, T.; Mayberry, W.; Smith, P. *J. Bacteriol.* **1974**, *119*, 106.
(57) De Rosa, M.; Gambacorta, A.; Bu'lock, J. D. *J. Gen. Microbiol.* **1975**, *86*, 156.
(58) Segerer, A.; Stetter, K. O. In *Bergey's Manual of Systemic Bacteriology*; Holt, J. G., Ed.; Williams and Wilkins: Baltimore, 1989; Vol. 3, pp 2250−2251.