NMR Studies of Substrate Binding to Cytochrome $P_{450\ BM3}$: Comparisons to Cytochrome $P_{450\ cam}$[†]

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ABSTRACT: The binding of the substrates sodium laurate and sodium 12-bromolaurate to the heme-containing domain of *Bacillus megaterium* cytochrome $P_{450\ BM3}$ (*CYP102*) has been studied by measurement of the relaxation effects of the unpaired electrons of the heme iron on the protons of water and of the bound substrates. Substrate binding leads to a conversion of the heme iron from a low-spin to a high-spin state, as shown by changes in the optical spectrum. The relaxation measurements show that this is accompanied by expulsion of water from the sixth coordination position of the iron, the distance between the iron and the water protons increasing from 2.6 to 5.2 Å. Corresponding relaxation measurements on the substrate protons lead to the determination of a number of distances between the iron and protons of the bound substrate and, hence, to information on the position and orientation of the substrate in the binding site. Laurate and 12-bromolaurate are found to bind in a very similar way, in an extended conformation with the carboxylate probably close to Arg47 and the other end of the chain 7.6−7.8 Å from the heme iron. It is shown that laurate and pyridine can bind simultaneously to the P_{450} domain and that the iron−laurate distances in this ternary complex are not significantly different from those in the binary complex. These observations are compared with those on the substrate complex of cytochrome $P_{450\ cam}$, and their implications for structural changes involved in the catalytic cycle are discussed.

The cytochrome P_{450}s constitute a superfamily of heme enzymes, currently with more than 200 members, which catalyze the monooxygenation of a wide variety of both exogenous and endogenous compounds through the insertion of one atom of molecular oxygen into the substrate with the concomitant reduction of the other atom to water. Members of this superfamily have been isolated from plants, bacteria, and yeast, as well as from mammals where they play a major role in determining the response of the organism to exogenous chemicals. Cytochrome P_{450}s are divided into two classes according to their redox partners. Class I cytochrome P_{450}s, found in the mitochondrial membrane of eukaryotes and in bacteria, are associated with an iron−sulfur protein which mediates electron transfer to the P_{450} from an NADH-dependent FAD- or FMN-containing reductase. The class II enzymes from the endoplasmic reticulum of eukaryotes, on the other hand, which are of most interest in the context of xenobiotic metabolism, require only a single redox partner, the flavoprotein NADPH−cytochrome P_{450} reductase.

Until recently, the only cytochrome P_{450} for which high-resolution structural information was available was cytochrome $P_{450\ cam}$ from *Pseudomonas putida*, a class I enzyme which catalyzes the 5-*exo*-hydroxylation of camphor (Poulos et al., 1986, 1987; Poulos & Raag, 1992; Raag et al., 1993, and references therein). Recently, a structure of a second class I enzyme, cytochrome $P_{450\ terp}$, has been determined (Hasemann et al., 1994). Studies of cytochrome $P_{450\ cam}$ have provided valuable insight into the mechanism of cytochrome P_{450}s [*e.g.*, Poulos et al. (1987), Raag et al. (1991), Poulos and Raag (1992), and Loida and Sligar (1993)], but its limited sequence similarity to the class II enzymes of the mammalian endoplasmic reticulum limits its usefulness as a model for all cytochrome P_{450}s.

The only well-characterized soluble class II enzyme is cytochrome $P_{450\ BM3}$ (*CYP102*) from *Bacillus megaterium*, which catalyzes hydroxylation of saturated and monounsaturated fatty acids, alcohols, and amides at the ω-1, ω-2, and ω-3 positions (Miura & Fulco, 1975; Ho & Fulco, 1976; Fulco, 1991). This is a unique catalytically self-sufficient P_{450}, having both a heme-containing P_{450} domain and a flavoprotein NADPH−cytochrome P_{450} reductase domain, which can be separately isolated after tryptic cleavage of the cytochrome $P_{450\ BM3}$ polypeptide chain. Both domains show clear sequence similarity to the corresponding mammalian microsomal enzymes (Ruettinger et al., 1989). Expression systems for the individual domains have been developed (Li et al., 1991; Oster et al., 1991; Miles et al., 1992); it has been reported that the monooxygenase activity of the intact $P_{450\ BM3}$ enzyme can be reconstituted using the individual domains, though the activity reported for the reconstituted system varies considerably (Boddupalli et al., 1992; Miles et al., 1992).

The X-ray crystal structure of the P_{450} domain of cytochrome $P_{450\ BM3}$ has recently been determined (Ravichandran

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et al., 1993). Comparison with the structure of cytochrome $P_{450\ cam}$ (Ravichandran et al., 1993; Li & Poulos, 1994; Hasemann et al., 1995) shows that although the overall topology is broadly similar (C_α rmsd of 2.8 Å), there are substantial differences between the two enzymes around the substrate binding pocket. While detailed information on substrate binding to $P_{450\ cam}$ is available [*e.g.*, Poulos et al. (1986, 1987), Raag and Poulos (1989, 1991), and Poulos and Raag (1992)], in the case of cytochrome $P_{450\ BM3}$ only the general identification of the substrate binding site has been reported (Hasemann et al., 1995). Comparison of the structures of cytochromes $P_{450\ cam}$, $P_{450\ terp}$, and $P_{450\ BM3}$ has shown that the substrate binding regions are the most variable parts of the structures and that the substrate binding site of $P_{450\ BM3}$ is appreciably larger than those of the other two enzymes (Hasemann et al., 1995).

We now report NMR studies of the interaction of lauric acid with the P_{450} domain[1] of cytochrome $P_{450\ BM3}$, in which we have used the paramagnetic relaxation effect of the unpaired electrons on the heme iron to obtain information on the location of the substrate in the binding pocket identified in the crystal structure. The results are compared with the information available on cytochrome $P_{450\ cam}$ and are discussed in terms of the mechanism of the enzyme.

MATERIALS AND METHODS

Materials. DEAE-Sephacel, PD10 columns, and Sephacryl S300 were from Pharmacia LKB. Hydroxyapatite resin was purchased from Bio-Rad Ltd. Deuterium oxide (100%) was purchased from Aldrich Ltd., [$^2H_5$]pyridine from Fluka, and isopropyl β-D-thiogalactopyranoside (IPTG) from NovoChem. Lauric acid (sodium salt), 12-bromolauric acid (sodium salt), and NADPH were purchased from Sigma Chemical Co. All other chemicals used were of at least analytical grade.

Protein Purification. The *Escherichia coli* plasmids pJM23, encoding the expression system for intact cytochrome $P_{450\ BM3}$, and pJM20, encoding the expression system for the P_{450} domain of $P_{450\ BM3}$ (Miles et al., 1992), were obtained from Dr. J. S. Miles, Department of Biochemistry, University of Glasgow, Glasgow, U.K. The host strain used was *E. coli* XL Blue 1 (*supE*44, *hsdR*17, *recA*1, *endA*1, *gyrA*46, *thi*, *relA*1, *lac*$^-$ F′ [*proAB*$^+$ *lacI*^{q} *lacZ*DM15 *Tn*10 (*tet*^{r})]). The intact enzyme and its P_{450} domain were expressed and purified to homogeneity essentially as described previously (Miles et al., 1992). The transformed cells were grown in Terrific Broth medium containing 50 μg/mL ampicillin and for pJM20 also in the presence of 50 μg/mL IPTG. The cells were harvested by centrifugation at 10 000 rpm for 15 min and the resuspended cells broken by passage twice through a French press (2.5 cm i.d. × 17 cm, Power Laboratory Press, American Instrument Co. Inc.). Cell lysates were fractionated by ammonium sulfate precipitation, followed by ion-exchange chromatography on a DEAE-Sephacel column, by chromatography on either an adenosine 2′-monophosphate agarose column for the intact cytochrome $P_{450\ BM3}$ or a hydroxyapatite column for the P_{450} domain, and finally, by gel filtration on a Sephacryl S300 column. This resulted in homogeneous proteins as shown by SDS−PAGE (with Coomassie blue staining), by electrospray mass spectrometry, and by the ratio of absorbance at 418 and 280 nm (where a ratio of ≥0.7 for intact cytochrome $P_{450\ BM3}$ or ≥1.7 for the P_{450} domain is characteristic of pure protein; A. W. Munro, personal communication). Protein concentrations were measured by the method of Omura and Sato (1964) using values of ϵ = 96 mM^{-1} cm^{-1} at 418 nm for intact cytochrome $P_{450\ BM3}$ and ϵ = 77.5 mM^{-1}cm^{-1} at 418 nm for the P_{450} domain. Fatty acid hydroxylation was measured spectrophotometrically by NADPH consumption as described by Matson et al. (1977), using an assay mixture containing 0.5 mM sodium laurate and 0.2 mM NADPH in 0.1 M phosphate buffer, pH 8.0.

NMR Spectroscopy. Proton NMR measurements were predominantly carried out at 500 MHz, using a Bruker AM500 spectrometer; studies of the frequency dependence of relaxation rates additionally involved measurements at 300 and 600 MHz. Samples contained 0.5−6.0 mM substrate and 45 μM to 3 mM P_{450} domain in 0.1 M phosphate buffer, pH* 8.0, in ^2H_2O. (The notation pH* refers to a pH meter reading uncorrected for isotope effects on the glass electrode.) All buffers were pretreated with Chelex 100 (Bio-Rad) to remove any traces of free metal ions. The sample temperature was 300 K unless otherwise specified. The longitudinal relaxation time (T_1) was measured by the inversion recovery method, using the (180°− τ −90°−acquire) pulse sequence (Hahn, 1950; Sass & Ziessow, 1977; Modi et al., 1989a,b). Proton chemical shifts were referred to the resonance of H^2HO as a secondary reference at 4.75 ppm.

Data Analysis. The longitudinal relaxation rate, $R_{1,obs}$ (=$1/T_{1,obs}$) was determined by fitting the measured peak height as a function of the interpulse delay, τ, to an exponential by nonlinear regression. Under fast-exchange conditions, this measured relaxation rate is the weighted average of the relaxation rates of the free and bound substrate ($R_{1,f}$ and $R_{1,b}$, respectively):

$$R_{1,obs} = p_f R_{1,f} + p_b R_{1,b} \quad (1)$$

where p_f (=$[S_f]/S_o$) and p_b (=$[S_b]/S_o$) are the fraction of the substrate in the free and bound state, respectively, and $[S_f]$, $[S_b]$, and S_o are the bound, free, and total substrate concentrations. Since under the conditions of the present experiments the substrate concentration is much greater than the protein concentration, $p_f \approx 1$ and

$$R_{1,obs} - R_{1,f} = \frac{E_o}{K_d + S_o}(R_{1,b} - R_{1,f}) \quad (2)$$

(assuming a stoichiometry of 1:1; see Results and Discussion), where E_o and S_o represent the total concentrations of the P_{450} domain and substrate, respectively, and K_d is the dissociation constant of the protein−substrate complex. In the present experiments, we wish to determine $R_{1,P}$, the paramagnetic contribution to the relaxation rate of the protons in the bound substrate due to the unpaired electrons of the heme iron. Each of the values of $R_{1,obs}$, measured using the paramagnetic protein, must therefore be corrected by subtracting the values, $R_{1,d}$, measured under the same conditions but with a diamagnetic control, in this case the reduced carbon monoxide complex of the P_{450} domain. Equation 2 then becomes

[1] For brevity, the term "P_{450} domain" will be used to refer to the heme-containing domain of cytochrome $P_{450\ BM3}$.

$$(R_{1,obs} - R_{1,d}) - R_{1,f} = \frac{E_o}{K_d + S_o}(R_{1,P} - R_{1,f}) \quad (3)$$

and by measuring $(R_{1,obs} - R_{1,d})$ as a function of protein and/or substrate concentration, estimates of K_d and $R_{1,P}$ can be obtained by fitting the data to eq 3.

For solvent relaxation

$$R_{1,P} = \frac{[H]}{E_o}(R_{1,obs} - R_{1,d}) = \frac{n}{T_{1,M} + \tau_m} = \frac{1}{T_{1,P}} \quad (4)$$

where [H] is the concentration of ^1H nuclei in the solvent, n is the number of exchangeable protons in the coordination sphere of the metal, and $T_{1,M}$ and τ_m are respectively their relaxation times and lifetimes. For fast exchange, $T_{1,M} \gg \tau_m$; the validity of this condition can be established by measuring the temperature dependence of $R_{1,P}$, since $T_{1,M}$ and τ_m have opposite dependences on temperature (Dwek, 1973).

The paramagnetic contribution, $R_{1,P}$, to the relaxation rate of the protons (of bound substrate or of water) arising from the unpaired electrons on the heme iron is related to the iron–proton distance by the Solomon–Bloembergen equation (Solomon & Bloembergen, 1956; Dwek, 1973). The assumptions underlying the use of this equation are discussed by Jardetzky and Roberts (1981); they include fast exchange (as above) and a negligible outer-sphere contribution to relaxation:

$$R_{1,P} = \frac{1}{T_{1,M}} = \frac{2}{15}\frac{\gamma_I^2 g^2 S(S+1)\beta^2}{r^6}\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_S^2\tau_c^2}\right) \quad (5)$$

where r is the distance of the proton from the heme iron, ω_I and ω_S are the nuclear and electronic Larmor frequencies, respectively, and τ_c is the effective correlation time of the dipolar interaction.[2] In this equation, we have retained only the term representing the dipolar interaction between the nuclear and the electron spins; the scalar term can be neglected (Dwek, 1973; Jardetzky & Roberts, 1981). In the case of cytochromes P_{450}, τ_c is determined by the electron spin relaxation time, τ_s (see Results and Discussion); order-of-magnitude arguments and measurements of $T_{1,P}$ and $T_{2,P}$ (Griffin & Peterson, 1975) indicate that the frequency dependence of τ_s can be neglected for cytochromes P_{450}.

Optical Spectroscopy. UV–visible spectra were obtained using a Beckman DU 650 spectrophotometer with quartz cells (10 mm path length) at 27 °C. Titrations were carried out by addition of substrates (20–400 μM) to the P_{450} domain (11.9 μM) in 0.1 M phosphate buffer (pH 8.0) in the sample cell and by diluting the same concentration of the domain in the reference cell with equal amounts of buffer. Equilibrium constants for substrate binding were calculated using the equation [*e.g.*, Sakurada et al. (1986), Modi et al. (1989c), and Saxena et al. (1990)]:

[2] In addition, following the usual conventions, γ_I is the magnetogyric ratio of the nucleus, g the isotropic g-factor, β the Bohr magneton, and S the spin quantum number of the electron.

Table 1: Substrate Binding and Catalysis by Cytochrome $P_{450\ BM3}$[a]

substrate	K_M (μM)	k_{cat} (s^{-1})	K_d (μM)
laurate	130 ± 10	25 ± 2	840 ± 40
12-bromolaurate	150 ± 10	19 ± 2	890 ± 50

[a] The kinetic parameters refer to the reaction catalyzed by the intact enzyme and the K_d values to binding to the cytochrome P_{450} domain, determined by optical spectroscopy. All experiments were carried out at 27 °C in 0.1 M phosphate buffer, pH 8.0.

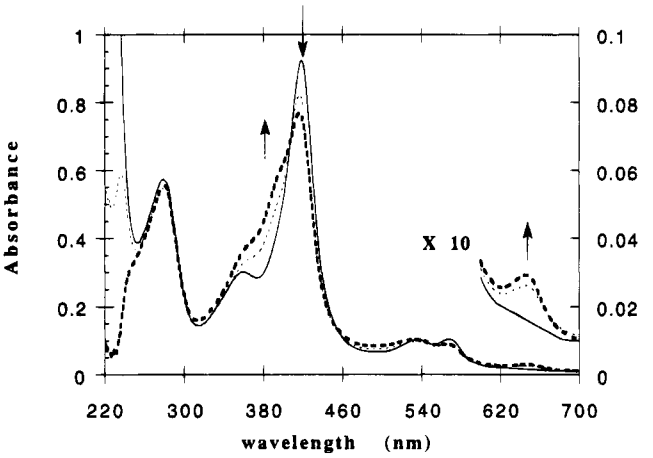

FIGURE 1: UV–visible spectra of the P_{450} domain of cytochrome $P_{450\ BM3}$ (11.9 μM) alone (—) and in the presence of sodium laurate [150 μM (• • •) and 300 μM (• • •)].

$$\frac{1}{\Delta A} = \left[\frac{K_d}{\Delta A_\infty}\right]\frac{1}{S_o} + \frac{1}{\Delta A_\infty} \quad (6)$$

where ΔA and ΔA_∞ are the changes in absorption (at 418 nm) at the substrate concentration S_o and at the saturating substrate concentration, respectively, and K_d is the dissociation constant of the enzyme–substrate complex. K_d and ΔA_∞ were estimated from the slope and intercept of a plot of $1/\Delta A$ *vs* $1/S_o$.

RESULTS AND DISCUSSION

The activity of cytochrome $P_{450\ BM3}$ in catalyzing the hydroxylation of sodium laurate was found to be characterized by a k_{cat} of 25 s^{-1} at pH 8.0 (Table 1), in good agreement with earlier reports (Li et al., 1991; Miles et al., 1992). The activity was found to be maximal between pH 7.5 and pH 8.5 (data not shown), and all spectroscopic experiments were therefore carried out at pH 8.0.

Figure 1 shows the electronic absorption spectra of the P_{450} domain and the effects of adding increasing concentrations of sodium laurate. It can be seen that the intensity of the Soret band at 418 nm decreases, while the intensity of bands at 390 and 650 nm increases. The dependence of these changes on the concentration of laurate can be used to estimate the equilibrium constant for substrate binding, using eq 6; the estimated K_d is given in Table 1. The band at 650 nm is characteristic of high-spin ferric heme proteins (Falk, 1964). Similar spectral changes on the formation of the complex of substrate with the Fe(III) form of the enzyme have been observed in other cytochromes P_{450} and have been attributed to a change in spin state of the heme iron from low spin ($S = {}^1/_2$) to high spin ($S = {}^5/_2$) (Dawson, 1988; Sariaslani, 1991). The value of ΔA_∞ obtained from analysis of the data using eq 6 is consistent with a complete spin-

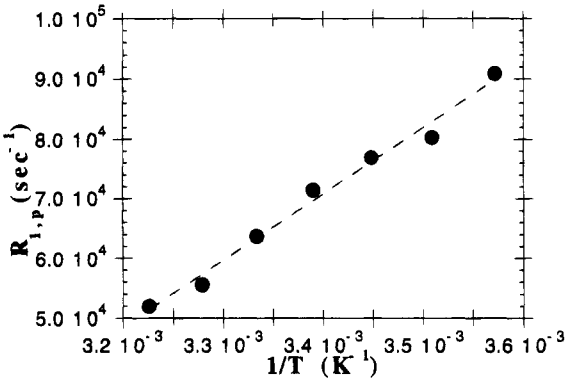

FIGURE 2: Temperature dependence of the paramagnetic contribution to the spin–lattice relaxation rate of water protons in the presence of the P_{450} domain of cytochrome $P_{450\ BM3}$ (3 mM); measurements were made at 600 MHz.

state conversion at saturating substrate concentrations. Direct evidence for a low-spin to high-spin conversion of cytochrome $P_{450\ BM3}$ on laurate binding has recently been obtained by electron spin resonance and resonance Raman spectroscopy (Miles et al., 1992).

It has also been shown in a number of other cytochromes P_{450} that a water molecule coordinated at the sixth position of the heme iron is expelled on binding of the substrate. The X-ray crystal structure of the P_{450} domain of cytochrome $P_{450\ BM3}$ shows that in this case water (or a hydroxide ion) is present in the sixth coordination position of the heme iron in the absence of substrate (Ravichandran et al., 1993). Measurement of the paramagnetic contribution to the relaxation rate of water protons can provide estimates of the distance of the nearest exchangeable proton from the heme iron [*e.g.*, Lanir and Schejter (1975), Philson et al. (1979), Jardetzky and Roberts (1981), Jacobs et al. (1987), and Modi et al. (1990)], although such experiments do not allow one to distinguish whether the exchange involves a whole water molecule, one or both of its protons, or an exchangeable proton on an amino acid residue.

The relaxation rate of the water protons was measured in solutions containing 45 μM to 3 mM concentrations of the P_{450} domain, in the presence and absence of substrate. The diamagnetic contribution to water proton relaxation was estimated by measurements using the reduced CO complex of the P_{450} domain. The water proton relaxation rate in the presence of the reduced CO complex was found to be identical to the relaxation rate in buffer, indicating that the diamagnetic contribution to relaxation was negligible. Use of this method to determine the distance between the iron and the nearest exchangeable proton requires (a) demonstration that the protons are exchanging rapidly in and out of the coordination sphere and (b) a value for τ_c, the correlation time of the dipolar interaction between the nuclear and electron spins. The temperature dependence of $R_{1,P}$ (Figure 2), showing a linear increase in $R_{1,P}$ as a function of reciprocal temperature, confirms that the fast-exchange condition is satisfied in the case of the P_{450} domain. The correlation time for the interaction was estimated by measuring $R_{1,P}$ at three frequencies (300, 500, and 600 MHz); fitting eq 5 to the values of $R_{1,P}$ as a function of ω_I^2 (data not shown) yields $\tau_c = 2.3\ (\pm 0.1) \times 10^{-10}$ s. This value is large compared to those reported for other low-spin ($S = {}^1/_2$) ferric heme proteins (Wüthrich, 1976) but comparable to those

Table 2: Relaxation Parameters and Derived Distances of Water Protons from the Heme Iron in Peroxidases and Cytochromes P_{450}

protein	$T_{1,P}$ (s)	τ_c (s)	S^a	r (Å)
horseradish peroxidase[b]	1.64×10^{-5}	9.5×10^{-11}	$^5/_2$	3.6
lactoperoxidase[b]	2.15×10^{-5}	4.5×10^{-10}	$^5/_2$	4.3
cytochrome $P_{450\ scc}$[c]	1.8×10^{-5}	3.5×10^{-10}	$^1/_2$	2.7
cytochrome $P_{450\ scc}$ + cholesterol[c]	2.6×10^{-5}	3.5×10^{-10}	$^5/_2$	3.9
cytochrome $P_{450\ cam}$[d]	0.42×10^{-5}	5.4×10^{-11}	$^1/_2$	2.6
cytochrome $P_{450\ cam}$ + camphor[e]	24.7×10^{-5}	3.0×10^{-10}	$^5/_2$	7.1
cytochrome $P_{450\ BM3}$ (P_{450} domain)	1.30×10^{-5}	2.3×10^{-10}	$^1/_2$	2.6
cytochrome $P_{450\ BM3}$ + lauric acid	7.14×10^{-5}	2.3×10^{-10}	$^5/_2$	5.2

[a] Spin state of the heme iron. [b] Modi et al., 1990b. [c] Jacobs et al., 1987. [d] Philson et al., 1979. [e] Griffin & Peterson, 1975.

reported for other cytochromes P_{450}. The average of all values reported for different cytochromes P_{450} (Griffin & Peterson, 1975; Philson et al., 1979; Woldman et al., 1985; Jacobs et al., 1987; Crull et al., 1989; Castro-Maderal & Sullivan, 1992) is 3×10^{-10} s. For these enzymes τ_c is determined by the electron spin–lattice relaxation time, τ_s, and it appears that a large τ_s value is a characteristic of cytochromes P_{450}.

Table 2 presents a comparison of the $T_{1,P}$ and τ_c values and exchangeable proton–iron distances for two cytochromes P_{450} and two peroxidases with the present data for the P_{450} domain of cytochrome $P_{450\ BM3}$. The iron–proton distances in $P_{450\ BM3}$ were calculated by assuming that the measured relaxation effect is due to two rapidly exchanging protons (of a water molecule); the distance of 2.6 Å in the absence of substrate estimated on this basis is entirely consistent with the iron–oxygen distance of 2.28 Å seen in the crystal structure (Ravichandran et al., 1993). This distance in $P_{450\ BM3}$ is also essentially identical to those estimated for $P_{450\ cam}$ and $P_{450\ scc}$; in solution there is clearly a water molecule in the first coordination sphere of the heme iron of cytochromes P_{450}, in contrast to the situation in the two peroxidases.

Addition of a 10-fold excess of substrate (lauric acid) to the P_{450} domain leads, as noted above, to a conversion to a high-spin state, but the water proton relaxation rate decreases (Table 2). The estimated distance of the closest exchangeable protons from the iron in the presence of lauric acid is 5.2 Å, again on the assumption that two water protons are involved.[3] This is clearly consistent with the idea that the water molecule is expelled from the coordination sphere of the heme iron on substrate binding. However, it is notable that in the P_{450} domain an exchangeable proton remains significantly closer to the heme iron in the substrate complex than is the case for cytochrome $P_{450\ cam}$, where a water proton–iron distance of 7–10 Å has been reported for the camphor complex from NMR relaxation measurements (Griffin & Peterson, 1975; Jacobs et al., 1987).

Measurements of the paramagnetic relaxation effects on the protons of the substrate molecule can be used in a similar way to obtain estimates of distances between individual protons of the bound substrate and the heme iron. Experiments of this kind have been carried out both with laurate and with 12-bromolaurate. The ^{1}H spectrum of laurate in aqueous solution (Figure 3) is not well resolved, separate resonances being seen only for the C2- and C3-methylene

[3] If only a single exchangeable proton is involved, its distance from the iron would be 4.6 Å.

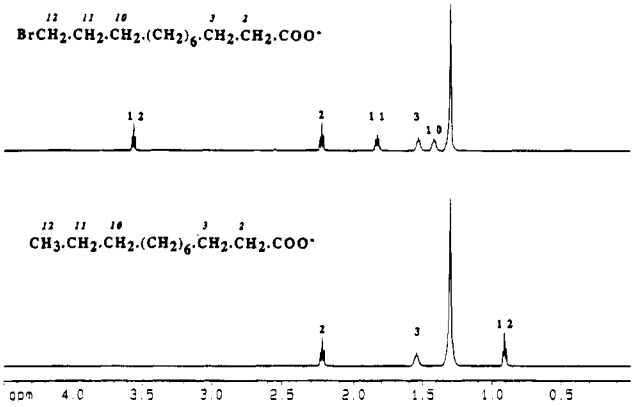

FIGURE 3: ^{1}H NMR spectra of laurate and 12-bromolaurate in 0.1 M phosphate buffer, pH 8, at 300 K; resonance assignments are indicated on the spectra.

groups and for the terminal methyl. 12-Bromolaurate was chosen in order to resolve the resonances of the C10- and C11-methylene groups as well (Figure 3). As shown in Table 1, in spite of the substantial additional bulk of the bromine atom, the kinetic parameters of 12-bromolaurate are virtually indistinguishable from those of laurate, and it has a very similar dissociation constant for binding to the P_{450} domain. Spin−lattice relaxation rates were measured at different substrate concentrations, with the concentration of the P_{450} domain held constant in a given experiment; because the relaxation effects of the heme iron on different substrate protons differ appreciably in magnitude, a series of experiments were carried out at different protein concentrations to permit precise measurements for all the resolved proton resonances. The temperature dependence of $R_{1,obs}$ of the C12 protons of 12-bromolaurate (which, along with the C12 protons of laurate, show the largest effect) at fixed substrate and protein concentrations (data not shown) established that the fast-exchange condition is satisfied under the conditions of these experiments. Figure 4 shows the values of $R_{1,obs}$ as a function of substrate concentration for the three resolved resonances of laurate and the five resonances of 12-bromolaurate. The curves are the best (least squares) fits of eq 3 to the data, which provide estimates of K_d and of $R_{1,P}$. The average values for K_d obtained from these experiments (880 μM for laurate and 820 μM for 12-bromolaurate) are in satisfactory agreement with those obtained from optical spectroscopy, confirming that exchange is rapid. It is difficult to determine the stoichiometry of binding from the relaxation experiments since the substrates bind only weakly, but fitting the data in Figure 4 to a version of eq 3 in which the stoichiometry is a variable gave an estimate of 1.0 (±0.3) molecules of substrate bound per P_{450} domain. The good agreement between the estimates of K_d from the relaxation effects on the substrate and the changes in heme absorbance does argue very strongly against the possibility that the substrate molecule closest to the heme is in slow exchange and the relaxation effects arise from a secondary binding site. To permit the calculation of the iron−proton distances from these $R_{1,P}$ values, τ_c was determined from the frequency dependence of $R_{1,P}$ of the C10, C11, and C12 protons of 12-bromolaurate; a mean value of 2.4 (± 0.3) × 10^{-10} s was obtained, the same within experimental error as that calculated from water proton relaxation (see above).

The $T_{1,M}$ values and estimated iron−proton distances are summarized in Table 3. These measurements show that the

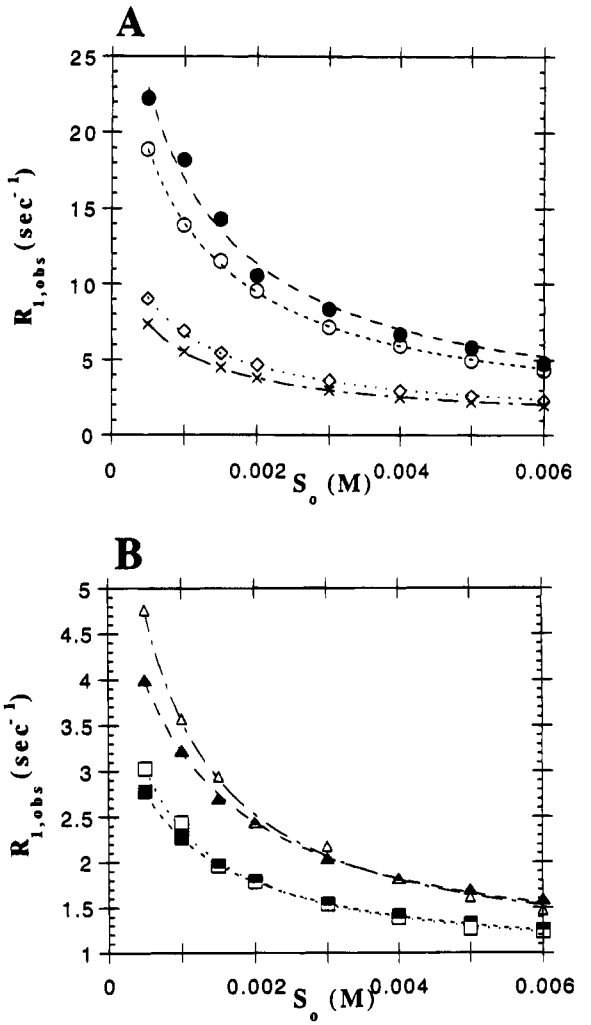

FIGURE 4: Measured spin−lattice relaxation rates for protons of the substrates sodium laurate and sodium 12-bromolaurate as a function of substrate concentration in the presence of the P_{450} domain of cytochrome $P_{450\ BM3}$: (A) 45 μM P_{450} domain; (B) 450 μM P_{450} domain. Solid symbols represent data for laurate and open symbols data for 12-bromolaurate: C2, □; C3, △; C10, ×; C11, ◇; C12, ○. Lines represent the least squares fit of eq 3 to the experimental data.

substrate adopts a rather extended conformation when bound to the enzyme, with the terminal $-CH_3$ or $-CH_2Br$ protons 7.6−7.8 Å from the heme iron and the C2-methylene protons 16.3−16.5 Å away. Equivalent protons in laurate and 12-bromolaurate are seen to be at the same distances from the iron, indicating that the two substrates bind to the enzyme in the same way. Figure 5 shows the active site of the P_{450} domain, taken from the crystal structure (Ravichandran et al., 1993), with lauric acid positioned within it on the basis of the limited number of measured distances available. Since there is as yet no information about any changes in the structure of the protein on substrate binding, this cannot be an accurate model of the initial enzyme−substrate complex, but it does illustrate two important points. First, the carboxylate group is positioned close to the flexible side chain of Arg47, consistent with the suggestion that an ion pair is formed between the guanidinium group of this residue and the substrate carboxylate (Ravichandran et al., 1993). The results of recent mutagenesis experiments in which Arg47 was replaced by a glutamate residue (C. Gibson, unpublished work) provide further support for this proposal.

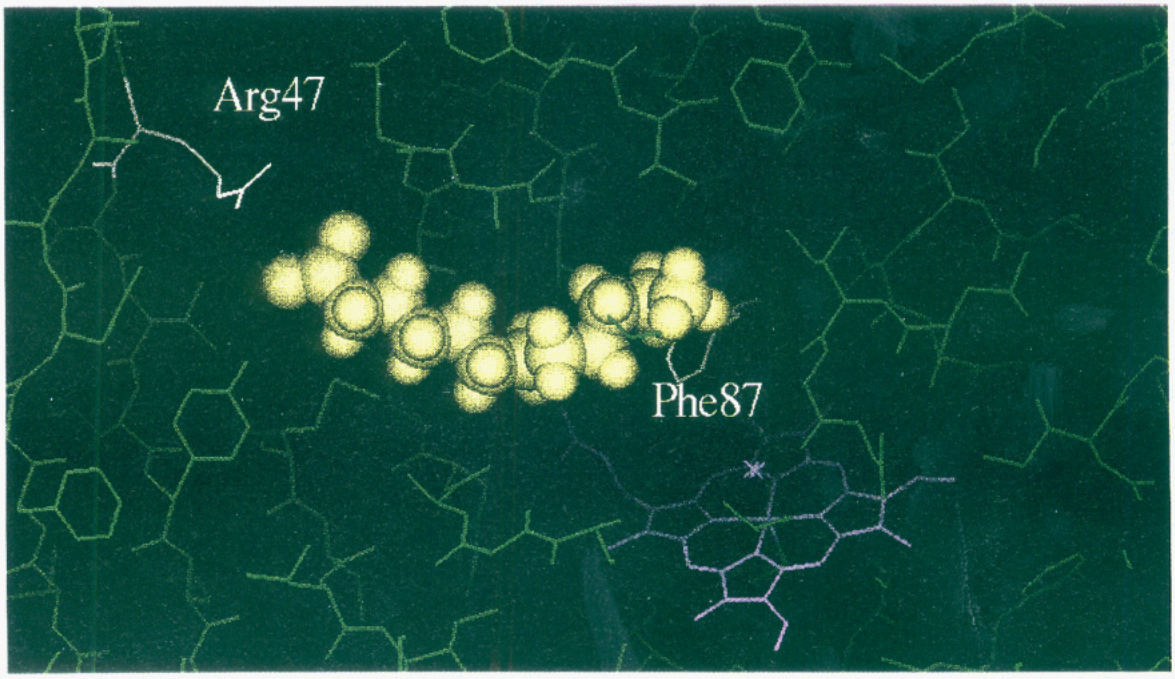

FIGURE 5: Substrate binding site of the P_{450} domain of cytochrome $P_{450\ BM3}$ (from the crystal structure; Ravichandran et al., 1993) with laurate positioned according to the measured distances from the heme iron listed in Table 3.

Table 3: Paramagnetic Relaxation Times and Distances from Protons of the Bound Substrate to the Heme Iron of the P_{450} Domain of Cytochrome $P_{450\ BM3}$

substrate	parameter	2 $-OOCCH_2-$	3 $-CH_2-$	10 $-CH_2-$	11 $-CH_2-$	12 $-CH_2X$[a]
laurate	$T_{1,M}$ (ms)	150 ± 10	99 ± 12	—[b]	—[b]	1.4 ± 0.3
	r (Å)	16.5 ± 0.2	15.4 ± 0.3			7.6 ± 0.3
12-bromolaurate	$T_{1,M}$ (ms)	140 ± 10	90 ± 11	5.1 ± 0.5	3.8 ± 0.4	1.7 ± 0.3
	r (Å)	16.3 ± 0.2	15.1 ± 0.3	9.4 ± 0.2	8.9 ± 0.2	7.8 ± 0.2
laurate + pyridine[c]	$T_{1,M}$ (ms)	163 ± 16	91 ± 7	—[b]	—[b]	1.3 ± 0.3
	r (Å)	16.7 ± 0.3	15.2 ± 0.2			7.5 ± 0.3

[a] X = H or Br. [b] Resonance not resolved. [c] 10-fold excess of pyridine over enzyme.

Second, the substrate is bound in this complex at some distance from the heme, the closest protons being 7.6−7.8 Å away. While there may be more than one mode of substrate binding (see below), the inverse sixth power distance dependence of the relaxation effects of the heme iron, together with the evidence outlined above demonstrating the absence of a slowly exchanging substrate molecule, means that these experiments will yield distances corresponding to the *closest* approach of the substrate to the heme.

In view of this observation, the relaxation experiment was repeated with laurate in the presence of 10 molar equiv of pyridine. Pyridine has been shown to bind very tightly to the heme iron of cytochromes P_{450} (Griffin & Peterson, 1972; Banci et al., 1994). In the presence of pyridine, the heme iron does not go from the low-spin to the high-spin state on addition of a large excess of laurate, since the substrate is unable to expel the strongly bound pyridine. The relaxation experiments gave an estimate of 960 μM for the dissociation constant of laurate in the presence of pyridine, only slightly higher than in its absence. As shown in Table 3, the distances of the laurate protons from the heme iron of the P_{450} domain are unaffected by the presence of pyridine. This is consistent with the position of laurate in the binding site shown in Figure 5, where there is sufficient space between the terminal methyl of laurate and the heme to accommodate a pyridine molecule.

These observations on the substrate complex of cytochrome $P_{450\ BM3}$ afford an interesting contrast to those reported for the corresponding complex of cytochrome $P_{450\ cam}$. The crystal structure of the camphor complex of $P_{450\ cam}$ shows the substrate bound in close proximity to the heme (Poulos et al., 1987), whereas laurate approaches no nearer than 7 Å to the heme iron of $P_{450\ BM3}$. It has recently been shown that pyridine and camphor cannot bind simultaneously to $P_{450\ cam}$ (Banci et al., 1994); the formation of a ternary complex with camphor has been suggested only for small molecules such as cyanide (Shiro et al., 1989). For $P_{450\ BM3}$, on the other hand, laurate and pyridine can bind at the same time, laurate binding in the same position and with very similar affinity in this ternary complex and in the binary complex.

The finding that, in the initial complex with the Fe(III) form of the P_{450} domain, laurate binds at some distance from the heme has implications for the mechanism of $P_{450\ BM3}$. First, substrate binding to this enzyme (either to the intact enzyme or to the P_{450} domain), as to other P_{450}s, leads to a low-spin to high-spin conversion of the heme iron, together with expulsion of water from the sixth coordination position. While in the case of $P_{450\ cam}$ this could be a direct effect, for $P_{450\ BM3}$ it is most likely to result from a structural change transmitted through the protein structure, since the substrate is too far away to have a direct effect.

Second, in this initial complex with the P_{450} domain laurate is too far away from the heme to be hydroxylated on the C9, C10, and C11 positions. Either the presence of the reductase domain in the intact enzyme[4] or electron transfer from this domain during the catalytic cycle must lead to a movement of the substrate closer to the heme, thus allowing direct transfer of the oxygen from the postulated peroxy intermediate to the substrate. It has been postulated that coupling of spin state, redox potential, and substrate binding in cytochromes P_{450} is achieved by desolvation of the substrate binding pocket (Poulos & Raag, 1992; Hasemann et al., 1995). It is clear from the relaxation measurements reported here that the heme environment is accessible to the solvent in the complex of the substrate with the oxidized state of cytochrome $P_{450\ BM3}$, and we suggest that desolvation accompanies the postulated movement of the substrate in the binding site, in a subsequent step of the catalytic cycle.

This postulated structural change would also have to be accompanied by a change in the substrate conformation, so that the ω-1, ω-2, and ω-3 positions would be close to the heme while the terminal methyl was positioned so as to be protected from hydroxylation. The X-ray crystal structure of the P_{450} domain shows that Phe87 makes van der Waals contact with the heme and is about 6 Å from the heme iron (Ravichandran et al., 1993). Our distance measurements show that the terminal methyl group of laurate is about 7.6 Å away from the iron and may contact Phe87 (see Figure 5). The postulated structural change may involve a movement of Phe87, and as suggested by Ravichandran et al. (1993), this residue may be involved in sequestering the ω-end of the substrate.

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[4] The presence of the reductase domain does not appear to have a major effect on the heme environment, since the optical absorption spectra of the heme, both in the "resting" state and in the reduced CO complex, are identical for the intact enzyme and the isolated P_{450} domain.

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