A Single Mutation in Cytochrome P450 BM3 Changes Substrate Orientation in a Catalytic Intermediate and the Regiospecificity of Hydroxylation†

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ABSTRACT: Phenylalanine 87 of *Bacillus megaterium* cytochrome P450 BM3, a residue close to the heme in the substrate binding pocket, has been replaced by alanine by site-directed mutagenesis. The substitution had no effect on the rate of hydroxylation of laurate and increased the affinity for laurate of both the intact enzyme and its heme domain by 2.6−6-fold in the ferric state. NMR paramagnetic relaxation measurements showed that in the initial ferric enzyme−substrate complex, where the substrate binds relatively far from the heme, the substitution had no effect on the position or orientation of the bound substrate. However, in the next intermediate in the catalytic cycle, the reduced enzyme, the position of the bound substrate was altered so that the terminal methyl group was 3.1 Å from the iron in the mutant, compared to 5.1 Å in the wild-type enzyme. Analysis of the products of the action of the enzyme on laurate and myristate showed that the mutant catalyzed hydroxylation almost exclusively at the ω position, in marked contrast to the wild-type enzyme, with which no hydroxylation at this position was observed.

The cytochromes P450 are widely distributed in animals, plants, fungi, and bacteria. They catalyze the insertion of one atom of oxygen into a wide range of substrates and in eukaryotes play a crucial role in drug and xenobiotic metabolism (Ortiz de Montellano, 1995). As yet, detailed structural information is only available for four soluble cytochromes P450 from bacteria (Poulos et al., 1987, 1995; Ravichandran et al., 1993; Hasemann et al., 1994, 1995; Cupp-Vickery & Poulos, 1995; Peterson & Graham-Lorence, 1995). Of these, cytochrome P450 BM3 from *Bacillus megaterium* (CYP102; Miura & Fulco, 1975; Narhi & Fulco, 1986; Wen & Fulco, 1987; Fulco, 1991; Ravichandran et al., 1993) appears to be the most closely related to the mammalian enzymes. It contains both a P450 heme domain and an NADPH−cytochrome P450 reductase domain in the same polypeptide chain, both of which show homology to the corresponding mammalian enzyme (Ruettinger et al., 1989). In particular, P450 BM3 catalyzes the hydroxylation of fatty acids at the ω-1, ω-2, and ω-3 positions, and its heme domain shows ∼25% homology to the mammalian class 4 P450s which also catalyze fatty acid hydroxylation, although predominantly at the ω position (CaJacob et al., 1988; Alterman et al., 1995; Bambal & Hanzlik, 1996).

In the crystal structure of the heme domain in the absence of substrate (Ravichandran et al., 1993), the substrate binding site is revealed as a large funnel-shaped opening extending from the molecular surface down to the heme and lined almost exclusively with hydrophobic residues. The only charged residue is Arg47, close to the molecular surface, which appears to be involved in binding the carboxylate group of the substrate (Modi et al., 1995a, 1996a; Li & Poulos, 1997; C. F. Oliver, S. Modi, W. U. Primrose, L.-Y. Lian, and G. C. K. Roberts, manuscript in preparation). In the initial complex of the substrate with the enzyme, in the ferric state, the substrate laurate binds some way from the heme (Modi et al., 1995a; Li & Poulos, 1997), but on reduction of the enzyme it moves closer to the heme into a position consistent with the observed hydroxylation at ω-1, ω-2, and ω-3 (Modi et al., 1996a).

The aromatic ring of phenylalanine 87 is in close contact with the heme on its distal side and perpendicular to it. Our model of substrate binding in the ferrous intermediate (Modi et al., 1996a) is consistent with the suggestion (Ravichandran et al., 1993) that this residue might play a role in determining the regiospecificity of the enzyme by "sequestering" the terminal methyl group of the substrate. Very recently, Capdevila et al. (1996) have reported that P450 BM3 metabolizes arachidonic acid stereoselectively to the 18(*R*)-hydroxy and 14(*S*),15(*R*)-epoxy derivatives and presented a model for the binding of this substrate to the enzyme in which Phe87 is proposed to play an important role in controlling the two modes of binding which lead to the two different products. We now report that substitution of this phenylalanine by alanine leads to a change in substrate binding, not in the initial enzyme−substrate complex but in an intermediate in the catalytic cycle, and changes the regiospecificity of the enzyme, converting it into an effective fatty acid ω-hydroxylase.

EXPERIMENTAL PROCEDURES

Hydroxyapatite resin was purchased from Bio-Rad Ltd., and DEAE Sephadex, Sephacel S300, and PD10 columns were from Pharmacia LKB Ltd. All restriction enzymes and

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their accompanying buffers were obtained from Gibco BRL Ltd. Isopropyl β-D-thiogalactopyranoside was from NovoChem; all other chemicals used were of at least analytical grade.

Protein Expression and Purification. Plasmids bearing the genes for P450 BM3 and its heme domain were obtained from Dr. J. Miles (Miles et al., 1992). Mutagenesis was performed using the Sculptor *in vitro* mutagenesis system (Amersham International plc) and confirmed by complete sequencing. Oligonucleotide synthesis and automated DNA sequencing were carried out by the Leicester Protein and Nucleic Acid Chemistry Laboratory. Proteins were expressed in *Escherichia coli* XL Blue 1 and purified to homogeneity essentially as described previously (Modi et al., 1995a). For purification of the intact enzyme, ion-exchange chromatography (HiLoad Q, Pharmacia) was used in place of affinity chromatography on a 2′,5′-ADP column. Yields of pure protein were 200−250 mg per liter of culture for the wild-type and 50−100 mg per liter of culture for the mutant. The proteins were shown to be homogeneous by SDS−PAGE, by electrospray mass spectrometry, and by the ratio of absorbance at 418 and 280 nm. Protein concentrations were determined by the method of Omura and Sato (1964) using ϵ_{418} values of 96 mM^{-1} cm^{-1} (intact enzyme) and 77.5 mM^{-1} cm^{-1} (heme domain).

Substrate Binding and Catalysis. Optical spectra were recorded on a Beckman DU650 spectrophotometer using 10 mm quartz cells at 27 °C. Equilibrium binding constants for laurate were determined from the changes in absorption at 418 nm as previously described (Modi et al., 1995a). Catalytic activity was measured spectrophotometrically by NADPH consumption (Matson et al., 1977), using laurate as substrate with 0.2 mM NADPH in 0.1 M phosphate buffer, pH 8.0. Production of hydrogen peroxide during the reaction was also measured by the method of Hildebrandt et al. (1977). The kinetic and dissociation constants were estimated by fitting a rectangular hyperbola to the data (typically four replicates at each of 9−10 substrate concentrations).

Product Identification. Reaction mixtures contained 1−6 mM substrate, 0.2−3 μM enzyme, and NADPH or an NADPH-regenerating system (3.3 mM glucose 6-phosphate, 1.3 mM $NADP^+$, 3.3 mM $MgCl_2$, and 0.4 unit/mL glucose-6-phosphate dehydrogenase) in 0.1 M phosphate, pH 8.0. For HPLC, the reaction was stopped after 3 h by acidification with HCl to pH 3.0−3.2, and the reaction mixtures were extracted twice with 20 mL of ethyl acetate. The pooled ethyl acetate layers were extracted with 4 mL of water, evaporated to dryness under nitrogen, and dissolved in 2 mL of 75% methanol. These extracts were analyzed by HPLC by a modification of the method of Okita et al. (1991), using a 0.46 cm × 25 cm C18 reverse phase S5 ODS2 column (Phase Separations) and eluting with methanol/0.2% aqueous acetic acid (38:62) for 30 min followed by 100% methanol for a further 30 min. One milliliter fractions were collected and identified by ^{1}H NMR spectroscopy; elution times were 14 (12-hydroxylaurate), 17 (11-hydroxylaurate), 19 (9 and 10-hydroxy-laurate), and 44−45 (laurate) min. Quantitative estimates of the ratios of products formed were obtained by integration of ^{1}H NMR spectra of the total reaction mixture or (for myristate) its ethyl acetate extract.

NMR Spectroscopy. Sample preparation, relaxation measurements (at 250, 300, 500, and 600 MHz) and data analysis were carried out as previously described (Modi et al., 1995a, 1996a,b). Samples contained 0.5−5.0 mM substrate and 2 μM to 4 mM P450 domain in 0.1 M phosphate, pH* 8.0, in ^2H_2O. (pH* indicates a pH meter reading uncorrected for isotope effects on the glass electrode.) The ferrous state of the heme domain was prepared in a glovebox under argon/nitrogen with a 5−10-fold excess of sodium dithionite. All buffers were pretreated with Chelex 100 (Bio-Rad) to remove any traces of free metal ions and were degassed and saturated with argon/N_2. The sample temperature was 300 K. The temperature dependence of relaxation confirmed that the fast exchange condition was satisfied. Iron−proton distances were calculated from the $T_{1,M}$ values by the Solomon−Bloembergen equation (Solomon & Bloembergen, 1956) as described earlier (Modi et al., 1995a). The correlation times required for this calculation were determined both from the T_1/T_2 ratio and from the frequency dependence of T_1 (over the range 250−600 MHz); the values obtained [2.3 (±0.1) × 10^{-10} s for the ferric state and 3.8 (±0.4) × 10^{-12} s for the ferrous state] were identical to those previously determined for the wild-type enzyme (Modi et al., 1995a, 1996a).

To produce Figure 2, the substrate molecule was positioned automatically into the crystal structure of the enzyme alone (Ravichandran et al., 1993) by the program MODELLER (Sali & Blundell, 1993), using constraints on the distances of substrate atoms from the iron derived from the paramagnetic relaxation experiments, together with a constraint to maintain the proposed ion pair between the guanidino group of Arg47 and the carboxylate of the substrate, in conjunction with simulated annealing and energy minimization (Modi et al., 1996a,b). Unless there was an overriding reason for a residue to move, to allow the constraints on the substrate position to be satisified, it remained in the same place due to its interactions in the crystal structure of the enzyme alone.

RESULTS AND DISCUSSION

The Phe87→Ala (F87A) mutants for both the intact enzyme and the P450 domain were characterized by sequencing and mass spectrometry, which gave results consistent with the expected structure. The heme environment in the mutants was characterized by optical spectroscopy; Figure 1 shows the spectra of the F87A mutant P450 domain. These are typical of a cytochrome P450; that is, in the oxidized state a spectrum with well-resolved α and β bands at 570 and 535 nm and a strong Soret band at 418 nm, while the spectrum of the CO complex of the reduced enzyme (Figure 1a) shows peaks at 547 and 448 nm. Closely similar spectra were obtained from the intact F87A enzyme, with the addition of a shoulder at around 460 nm arising from the flavins. The absence of any band at 420 nm in the CO complex of the reduced enzyme shows that there is no inactive P450 in the preparation (Modi et al., 1995b). Comparison with the spectra of the wild-type enzyme [not shown; see Miles et al. (1992) and Modi et al. (1995a)] shows that, although this phenylalanine side chain is in contact with the heme, substitution by an alanine does not significantly affect the electronic structure of the heme as reflected in its optical spectrum. Figure 1b shows the effects of addition of the substrate laurate; a typical "type I" spectrum is produced, characterized by a decrease in the Soret band at 418 nm and the α and β bands at 570 and 535 nm, respectively, with a simultaneous increase in the absorbance at 390 and 650 nm. The band at 650 nm is characteristic of high-spin ferric heme proteins (Falk, 1964), and these effects

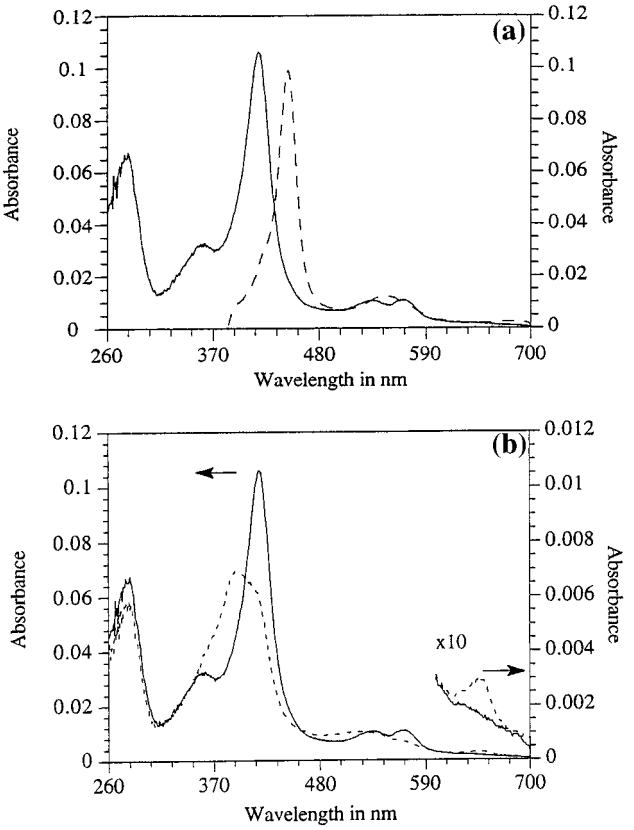

FIGURE 1: Optical spectra of the F87A mutant of cytochrome P450 BM3. (a) The F87A P450 domain (1.4 μM) in the resting (ferric) state (solid line) and in the carbon monoxide complex of the reduced enzyme (dashed line). (b) The F87A P450 domain (1.4 μM) alone (solid line) and in the presence of 300 μM lauric acid (dashed line).

Table 1: Laurate Binding and Hydroxylation by Wild-Type and Mutant Cytochrome P450 BM3[a]

	K_d (μM)				
protein	P450 domain	intact enzyme	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (M^{-1} s^{-1})
wild-type	840 ± 40	270 ± 47	26 ± 1	136 ± 3	1.9×10^5
F87A mutant	140 ± 44	103 ± 53	25 ± 4	167 ± 50	1.5×10^5

[a] The dissociation constants refer to binding to the ferric state of the enzyme.

of addition of substrate (Dawson, 1988; Sariaslani, 1991) have been attributed to a change in spin state of the heme iron from low spin ($S = {}^1/_2$) to high spin ($S = {}^5/_2$). The change in the spectrum at saturating laurate concentrations is consistent with a complete spin-state conversion, as seen in the wild-type enzyme (Modi et al., 1995a).

The affinity for laurate of both the P450 domain and the intact enzyme in the ferric state is increased 2.6−6-fold by the F87A substitution (Table 1). The K_M for laurate, which may reflect binding to the reduced rather than the resting state of the enzyme (Modi et al., 1996a), is essentially the same for wild-type and F87A enzyme, as is the k_{cat} value measured by NADPH consumption (Table 1). To assess the coupling of NADPH consumption to substrate hydroxylation, we determined the amount of peroxide produced; this was negligible ($<10^{-9}$ mol of peroxide produced per mole of NADPH consumed) for both wild-type and mutant enzyme. This substitution thus has very little effect on the overall catalytic efficiency of the enzyme, as measured by k_{cat}/K_M values (Table 1).

In order to make a structural comparison of substrate binding to the wild-type and mutant enzyme, we have estimated distances between the individual protons of the bound substrate and the heme iron in the P450 domain of the enzyme from the paramagnetic relaxation effects of the iron, using both laurate and 12-bromolaurate. (The latter, which is as good a substrate as laurate, has a better resolved ^{1}H NMR spectrum; Modi et al., 1995a.) The necessary controls to ensure rapid exchange of the substrate between bound and free states, to correct for diamagnetic contributions to relaxation, and to determine the relevant correlation times were carried out as described in detail by Modi et al. (1995a). The paramagnetic contributions to the relaxation times of the bound substrate and the derived iron−proton distances are given in Table 2, where the distances are compared with those for the wild-type enzyme.

The initial enzyme−substrate complex is formed with the ferric state of the enzyme. In this complex the substrate is relatively distant from the heme (and from Phe87), the closest approach being that of the terminal methyl group, whose protons are 7.6−7.8 Å from the heme iron (Modi et al., 1995a). The position and orientation of both laurate and 12-bromolaurate bound to the ferric state of the F87A mutant are essentially the same as for the wild-type enzyme (Table 2).

The next intermediate in the catalytic cycle is the ferrous enzyme−substrate complex, formed by the transfer of one electron from the reductase. We have shown (Modi et al., 1996a) that this electron transfer is accompanied by a substantial movement of the substrate (and presumably a structural change of the protein), such that the ω-1 and ω-2 methylene groups move some 6 Å closer to the iron, into a position appropriate for hydroxylation. The terminal (ω) methyl group, which is not hydroxylated by the wild-type enzyme, is 5.1 Å from the iron in the ferrous intermediate of the wild-type enzyme (Table 2) and is close to Phe87 in our model of the complex (Figure 2). In the complex with the ferrous state of the F87A mutant, while the positions of the ω-1 and ω-2 methylenes are unaffected, the terminal methyl group is clearly closer to the iron than that in the wild-type enzyme (3.1 *vs* 5.1 Å; Table 2 and Figure 2). Thus this amino acid substitution has no effect on substrate binding in the initial enzyme−substrate complex but does significantly alter the conformation of the bound substrate in the subsequent catalytic intermediate.

This difference in the position of the terminal methyl group suggested that the mutant might, unlike the wild-type, hydroxylate the substrate at this position. We therefore identified and quantified the products of hydroxylation of laurate and myristate. The products were initially identified by NMR of fractions from HPLC of extracts of the reaction mixture; Figure 3 shows the ^{1}H NMR spectra of laurate and the isomeric hydroxylaurates isolated in this way. In order to obtain an accurate quantitation of the relative amounts of each product formed, we integrated the ^{1}H NMR spectra of the reaction mixtures. Figure 4 shows the spectra of the reaction mixture after incubation of wild-type and F87A mutant P450 BM3 with laurate and NADPH. Protons geminal to the hydroxyl group appear at 3.5−3.9 ppm; a clear triplet from the $-C(12)H_2OH$ of ω-hydroxylaurate appears at 3.57 ppm in the incubation with the F87A enzyme (Figure 3b) but not in that with the wild-type (Figure 3a). The region 0.8−1.2 ppm contains resonances from the

Table 2: Paramagnetic Relaxation Times and Distances of Substrate Protons from the Heme Iron in the Ferric and Ferrous States of the Heme Domain of Wild-Type and F87A Mutant Cytochrome P450 BM3

substrate	protein	parameter	C2 $^{-}OOCCH_2-$	C3 $-CH_2-$	C10 $-CH_2-$	C11 $-CH_2-$	C12 $-CH_2X$[a]
ferric state[b]							
laurate	F87A mutant	$T_{1,M}$ (ms)	130 ± 2	80 ± 3	*d*	*d*	1.29 ± 0.03
		r (Å)	16.9 ± 0.2	15.6 ± 0.2	*d*	*d*	7.8 ± 0.3
	wild-type[c]	*r* (Å)	16.5 ± 0.2	15.4 ± 0.3	*d*	*d*	7.6 ± 0.3
bromolaurate	F87A mutant	$T_{1,M}$ (ms)	128 ± 1	78 ± 1	3.1 ± 0.1	2.23 ± 0.08	1.18 ± 0.06
		r (Å)	16.9 ± 0.1	15.5 ± 0.1	9.1 ± 0.2	8.6 ± 0.2	7.7 ± 0.2
	wild-type[c]	*r* (Å)	16.3 ± 0.2	15.1 ± 0.3	9.4 ± 0.2	8.9 ± 0.2	7.8 ± 0.2
ferrous state[b]							
laurate	F87A mutant	$T_{1,M}$ (ms)	*e*	*e*	*d*	*d*	0.43 ± 0.1
		r (Å)	*e*	*e*	*d*	*d*	3.15 ± 0.6
	wild-type[c]	*r* (Å)	*e*	*e*	*d*	*d*	5.1 ± 0.2
bromolaurate	F87A mutant	$T_{1,M}$ (ms)	*e*	*e*	0.25 ± 0.05	0.38 ± 0.1	0.40 ± 0.05
		r (Å)	*e*	*e*	2.9 ± 0.06	3.1 ± 0.07	3.1 ± 0.05
	wild-type[c]	*r* (Å)	*e*	*e*	3.0 ± 0.1	3.1 ± 0.1	5.1 ± 0.1

[a] X = H or Br. [b] Relaxation measurements on the ferric state were carried out at 250 MHz, and those on the ferrous state were carried out at 600 MHz. [c] Data from Modi et al. (1995a, 1996a). [d] Resonance not resolved. [e] Insufficient paramagnetic contribution to relaxation for a distance to be estimated.

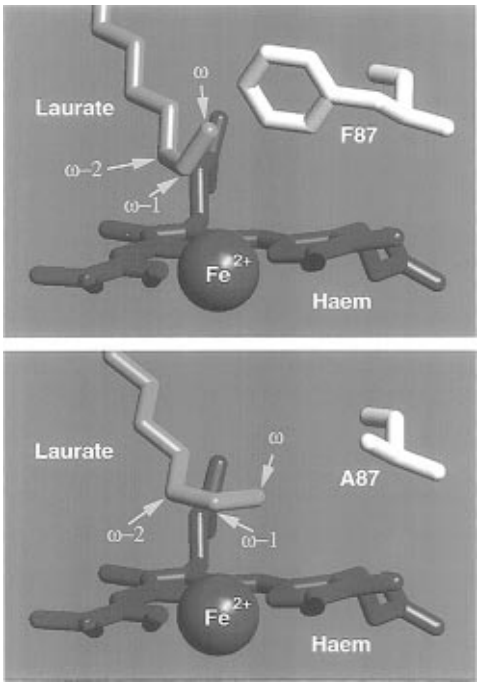

FIGURE 2: Model of the binding of laurate to the wild-type (top) and F87A mutant (bottom) cytochrome P450 BM3 in the ferrous state of the complex. The substrate was docked into the crystal structure of the heme domain of the enzyme on the basis of the iron−proton distance constraints derived from the paramagnetic relaxation experiments (Table 2). In the absence of detailed structural information on the ferrous state of the enzyme−substrate complex, these structures should be regarded only as an indication of the position and orientation of the substrate in the binding site of the wild-type and mutant enzymes.

$-C(12)H_3$ in the substrate and in the ω-1, ω-2, and ω-3 hydroxylated products; these are clearly present in Figure 3a but are very weak in Figure 3b, showing that the F87A mutant produces only very small amounts of these products. Resonances from methylene protons vicinal to a secondary hydroxyl group appear at 1.3−1.5 ppm in Figure 3a, but are clearly absent in Figure 3b. The resonance from the $C(11)H_2$ of ω-hydroxylaurate appears at 1.51 ppm in Figure 3b, coincident with that of the invariant $C(3)H_2$. The identity of the products was confirmed by HPLC, and their relative amounts were determined both by HPLC and by integration of spectra such as that shown in Figure 3, using the invariant $C(2)H_2$ resonance as a standard. For the wild-type enzyme acting on laurate we observed a 0:30:70 ratio of ω:ω-1:(ω-2 + ω-3) hydroxylated products [in agreement with Miura and Fulco (1975)]; no detectable ω-hydroxylated product was observed. By contrast, the F87A mutant hydroxylated laurate and myristate almost entirely (>90%) at the ω position.

The regiospecificity of wild-type cytochrome P450 BM3, for hydroxylation at the internal methylene carbons and not the terminal methyl carbon, corresponds to the relative chemical reactivity at these positions; primary C−H bonds are stronger than secondary C−H bonds. However, the regiospecificity is in fact actively determined by the enzyme. For example, substrates having terminal double or triple bonds, which are intrinsically more easily oxidized, are hydroxylated by P450 BM3 almost exclusively at the ω-2 position, with no more than a trace of oxidation of the terminal π bond (Shirane et al., 1993). A number of mammalian cytochromes P450 which hydroxylate fatty acids are relatively nonspecific, but P450 4A1, which shows ~25% identity to P450 BM3, catalyzes hydroxylation predominantly (>80%) at the chemically less favorable ω-position (CaJacob et al., 198; Alterman et al., 1995; Bambal & Hanzlik, 1996); in this enzyme the residue corresponding to Phe87 in P450 BM3 is a leucine (Hasemann et al., 1995). In the present case, a point mutation has converted the enzyme from one which specifically suppresses hydroxylation at the ω position to one which specifically favors hydroxylation at this chemically less favorable position. This has not arisen from a simple change in the position of the substrate in the initial enzyme−substrate complex, since the mutation has no effect on this. There is a clear difference in substrate orientation in the next, reduced, intermediate, but even in this intermediate, the iron−proton distances, which are essentially equal for the ω, ω-1, and ω-2 carbons (Table 2), do not explain the observed preference for ω-hydroxylation in the mutant. Energy minimization and molecular dynamics simulations of P450 BM3 in the absence of substrate suggest that the active-site cleft can undergo substantial conformational changes (Li & Poulos, 1995; Paulsen & Ornstein, 1995). The movement of the bound substrate on reduction of the protein (Modi et al., 1996a) provides indirect evidence for a structural change accompanying the transfer of the first electron in the catalytic cycle. The comparison of the substrate position in the reduced F87A enzyme with the observed regiospecificity

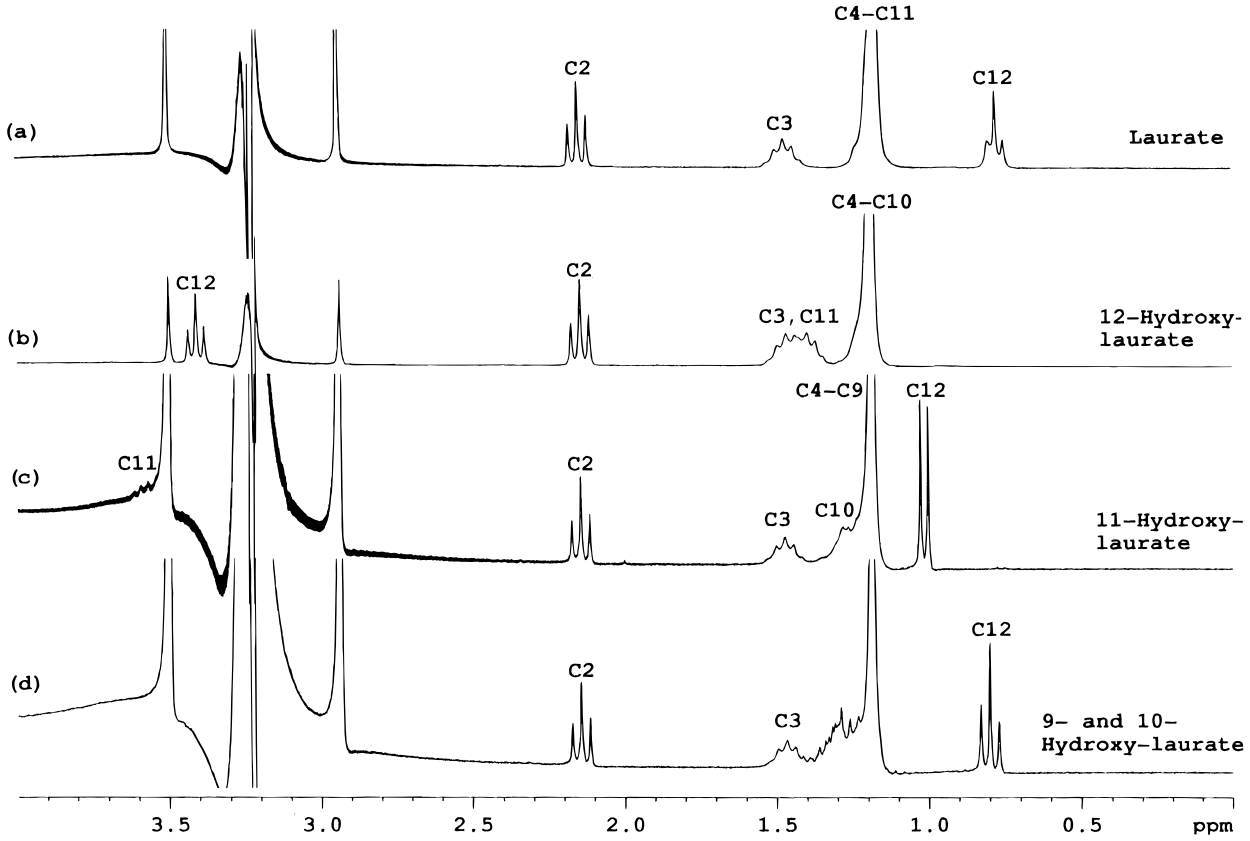

FIGURE 3: 250 MHz ^{1}H NMR spectra (CD_3OD, 300 K) of the HPLC-purified products of enzymic hydroxylation of lauric acid. (a) Lauric acid substrate, (b) 12-hydroxylauric acid, (c) 11-hydroxylauric acid, and (d) mixture of 9- and 10-hydroxylauric acid (not separated under the HPLC conditions used). Resonance assignments are indicated on the spectra.

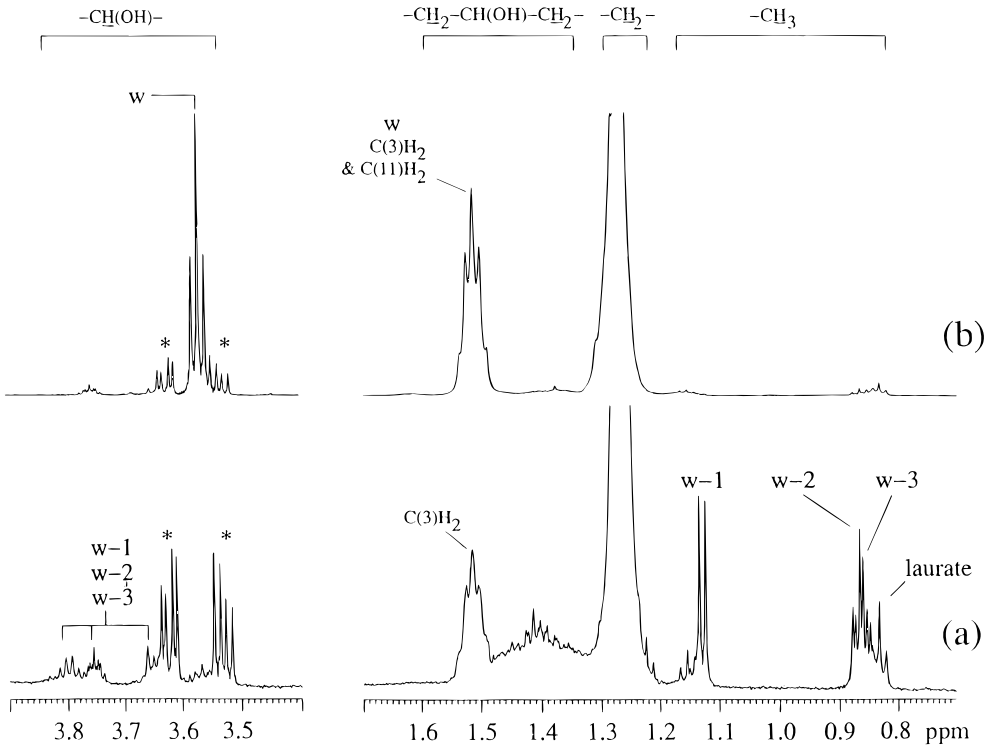

FIGURE 4: 600 MHz ^{1}H NMR spectra of the reaction mixtures from incubations of (a) wild-type and (b) F87A mutant P450 BM3 (each at 0.2 μM) with 1 mM laurate and 1 mM NADPH. The intensities of the spectra were normalized using the invariant resonance of the C2 protons (2.20 ppm). Resonance assignments are indicated on the spectra; signals from $NADP^+$ at 3.52 and 3.62 ppm are indicated by asterisks.

suggests that, in order to account for the almost exclusive ω-hydroxylation, additional structural changes in subsequent steps must be postulated. There appears to be a progressive adaptation of the active site of cytochrome P450 BM3 through the catalytic cycle, which goes beyond the expected accommodation of the changing structure of the heme in

successive intermediates, to a series of progressive changes in the position and orientation of the substrate. In this enzyme, and perhaps in others, the regiospecificity of catalysis is not simply determined by the geometry of the initial enzyme−substrate complex but by a series of structural changes extending through much of the catalytic cycle.

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