

Engineering the substrate specificity of *Bacillus megaterium* cytochrome *P*-450 BM3: hydroxylation of alkyl trimethylammonium compounds

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Oligonucleotide-directed mutagenesis has been used to replace arginine-47 with glutamate in cytochrome *P*-450 BM3 from *Bacillus megaterium* and in its haem domain. The mutant has been characterized by sequencing, mass spectrometry, steady-state kinetics and by optical and NMR measurements of substrate binding. The mutant retains significant catalytic activity towards C_{12} – C_{16} fatty acids, catalysing hydroxylation in the same ($\omega-1$, $\omega-2$, $\omega-3$) positions with k_{cat}/K_m values a factor of 14–21 lower. C_{12} – C_{16} alkyl trimethylammonium compounds are relatively poor substrates for the wild-type enzyme, but are efficiently hydroxylated by the arginine-47 \rightarrow glutamate mutant at the $\omega-1$, $\omega-2$ and $\omega-3$ positions, with k_{cat} values of up to 19 s^{-1} . Optical spectroscopy shows that the binding of the C_{14} and C_{16} alkyl

trimethylammonium compounds to the mutant is similar to that of the corresponding fatty acids to the wild-type enzyme. Paramagnetic relaxation measurements show that laurate binds to the ferric state of the mutant in a significantly different position, 1.5 Å closer to the iron, than seen in the wild-type, although this difference is much smaller ($\sim 0.2\text{ Å}$) in the ferrous state of the complex. The binding of a substrate having the same charge as residue 47 to the ferric state of the enzyme is roughly ten times weaker than that of a substrate having the opposite charge (and thus is able to make an ion-pair interaction with this residue). The results are discussed in the light of the three-dimensional structure of the enzyme.

INTRODUCTION

The cytochrome *P*-450s catalyse the mono-oxygenation of a wide variety of compounds through the insertion of one atom of molecular oxygen into the substrate, with the concomitant reduction of the other atom to water. The members of this family, which occur in the endoplasmic reticulum of mammals, play a central role in determining the response of the organisms to foreign chemicals, both therapeutic drugs and environmental contaminants. As yet, no three-dimensional structure is available for a mammalian *P*-450, but crystal structures have been reported for four soluble bacterial enzymes of this family [1–6]. Of these, cytochrome *P*-450 BM3 from *Bacillus megaterium* A.T.C.C. 14581, the structure of whose haem domain was reported by Ravichandran et al. [3], is of particular interest as a soluble model for the membrane-bound mammalian enzymes. *P*-450 BM3 (CYP102) catalyses hydroxylation (in the $\omega-1$, $\omega-2$ and $\omega-3$ positions) and/or epoxidation of medium- and long-chain fatty acids [7–11]. It contains a *P*-450 haem domain and an NADPH-cytochrome *P*-450 reductase flavoprotein domain in a single polypeptide chain (M_r 118 000), both of which show clear sequence homology with the corresponding mammalian proteins [12]. In addition to its importance as a model for the mammalian enzymes, the fact that this is a ‘self-contained’ soluble *P*-450 makes it attractive for applications in organic synthesis.

The structure of the haem domain of *P*-450 BM3 [3] reveals a large hydrophobic funnel-shaped opening, approximately 20 Å long and 10 Å in diameter, extending from the surface of the molecule down to the haem. Fatty-acid substrates initially bind relatively far from the haem, the terminal methyl being approx. 7.5 Å from the haem iron [13,14]. On reduction of the enzyme–substrate complex there is a conformational change which leads to a 6 Å movement of the substrate towards the haem, into

position for hydroxylation at the $\omega-1$, $\omega-2$ and $\omega-3$ positions [15].

The roles of two residues in this large substrate-binding pocket have been explored by site-directed mutagenesis. The aromatic ring of phenylalanine-87 is close to the haem on the distal side, and it was suggested that this residue could be important in sequestering the terminal methyl of a fatty-acid substrate, thus preventing hydroxylation at this position [3]. We have recently shown that replacement of this residue with an alanine converts the enzyme into a regiospecific ω -hydroxylase for saturated fatty acids [16], while Graham-Lorence et al. [17] showed that a phenylalanine to valine mutation at this position similarly increased the regiospecificity of the enzyme towards arachidonic acid, making it a specific (14*S*,15*R*)-epoxygenase for this substrate. The only charged residue in the substrate-binding site is arginine-47, which is located at the opening of the substrate access channel, and which has been proposed to be involved in binding the carboxylate group of the substrate. Graham-Lorence et al. [17] have reported that the arginine-47-to-glutamate mutant is inactive towards arachidonic acid. In this paper, we show that this mutant retains significant hydroxylase activity towards saturated fatty acids and shows much increased activity towards C_{12} – C_{16} alkyl trimethylammonium compounds.

EXPERIMENTAL

Chemicals and reagents

Restriction enzymes, with their accompanying buffers, were from Gibco-BRL Ltd. Isopropyl- β -D-thiogalactopyranoside was from NovoChem, and hydroxyapatite resin from Bio-Rad Ltd. DEAE-Sephadex, PD10 columns and Sephacel S300 were obtained from Pharmacia LKB Ltd. Substrates were obtained from Aldrich

Abbreviations used: C_{12} TMA, dodecyltrimethylammonium bromide; C_{14} TMA, tetradecyltrimethylammonium bromide; C_{16} TMA, hexadecyltrimethylammonium bromide.

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Chemical Company Ltd. or Sigma Chemical Co., and all other chemicals used, were of at least analytical grade, were from Sigma Chemical Co. or from Fisons Ltd.

Bacterial strains and vectors

For DNA manipulations or protein expression the *Escherichia coli* strains used were either TG1 [*supE hsd5 thi lac⁺ proAB F'* (*traD36 proAB⁺ lacI^a lacZM15*)] or XL Blue 1 [*supE44 hsdR17 recA1 gyrA46 thi relA1 lac⁻ F'* [*proAB⁺ lacI^a lacZM15 Tn10 (tet^r)*]]. Expression plasmids containing either the complete *CYP102* gene from *B. megaterium* or a 1.5 kb segment encoding residues 1–472 (the haem domain) of *P-450 BM3* [18] were obtained from Dr. J. Miles, Department of Biochemistry, University of Glasgow, Glasgow, U.K.

Site-directed mutagenesis

Oligonucleotide-directed mutagenesis was performed using the Sculptor *in vitro* mutagenesis system from Amersham International plc. Oligonucleotides were synthesized using Applied Biosystems 380B or 394 synthesizers, and automated DNA sequencing was performed using the PRISM ready reaction DyeDeoxy Terminator cycle sequencing kit on an Applied Biosystems 373A DNA Sequencer (Leicester Protein and Nucleic Acid Chemistry Laboratory, Hodgkin Building, Lancaster Road, Leicester LE1 9HN, U.K.). DNA manipulations and bacterial transformations were performed by standard methods [19].

Expression and purification of wild-type and mutant *P-450 BM3* and its haem domain

Intact *P-450 BM3* and its haem domain were expressed and purified to homogeneity as described previously [13,16]. The wild-type proteins were expressed in *E. coli* XL Blue 1 [13,16], and the mutant proteins in *E. coli* TG1. The purified proteins were shown to be homogeneous by SDS/PAGE (Coomassie Blue staining), by electrospray mass spectrometry (VG Instruments) and by the ratio of absorbance at 418 nm and 280 nm. Protein concentrations were measured by the method of Omura and Sato [20], using values of $\epsilon = 96 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 418 nm for intact cytochrome *P-450 BM3* and $\epsilon = 77.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 418 nm for the haem domain.

Enzyme assays and substrate binding

Hydroxylation of fatty acids and alkyl trimethylammonium compounds was measured spectrophotometrically by NADPH consumption as described by Matson et al. [21], using an assay mixture containing substrate and 0.2 mM NADPH in 0.1 M phosphate buffer, pH 8.0. The production of hydrogen peroxide during the hydroxylation reaction was determined by the method of Hildebrandt et al. [22]. Equilibrium constants for substrate binding to the ferric form of the enzyme were determined from the changes in absorbance at 418 nm as described previously [13]. Both kinetic and binding data were analysed by non-linear regression using Enzfitter 1.05 (Elsevier-Biosoft). For some of the short-chain substrates, K_d values were not easily determined by direct optical titration, since they were comparable with the critical micelle concentrations. In these cases, K_d values were estimated by competition with a reference substrate of known K_d . The observed dissociation constant, $K_{d,\text{obs}}$, of the reference substrate in the presence of a concentration, $[S_2]$, of the test substrate is given by:

$$K_{d,\text{obs}} = K_{d,1} + \frac{K_{d,1}[S_2]}{K_{d,2}} \quad (1)$$

(see, e.g., [23]), where $K_{d,1}$ and $K_{d,2}$ are the intrinsic dissociation constants of the reference and test substrates respectively.

NMR spectroscopy

Proton NMR measurements were carried out at 250 MHz or 600 MHz using Bruker AR250 or AMX600 spectrometers. Studies of the frequency dependence of the relaxation rates additionally involved measurements at 300 and 500 MHz. For identification of the products of the enzyme activity, spectra of the reaction mixture were obtained as described by Oliver et al. [16]. For measurements of the paramagnetic relaxation of substrate protons by the haem iron, samples contained 0.5 to 6.0 mM substrate, 45 μM to 3 mM haem domain [either wild-type or arginine-47 \rightarrow Glu (R47E) mutant] and 0.1 M phosphate buffer, pH* 8.0, in $^2\text{H}_2\text{O}$. (The notation pH* indicates a pH meter reading uncorrected for the deuterium isotope effect on the glass electrode.) The ferrous state of the haem domain was prepared in a glove box under argon/nitrogen with a 5–10 fold excess of sodium dithionite, as described previously [15]. The sample temperature was 300 K unless otherwise stated. The longitudinal relaxation rates of substrate protons were measured as a function of substrate concentration and the data were analysed as described previously [13].

RESULTS

Protein expression and characterization

Arginine-47 was replaced with glutamate both in the intact cytochrome *P-450 BM3* and in its haem domain. In both cases, yields of the arginine-47 \rightarrow Glu (R47E) mutant proteins were comparable with those of the wild-type, the range was 200–250 mg of pure protein/litre of culture. All proteins were pure, shown by the resolution into a single band on a SDS/polyacrylamide gel stained with Coomassie Blue, and by the absorbance ratio of A_{418}/A_{280} . N-terminal sequences were obtained for the first 18 residues of all four proteins, confirming the purity of the preparations. The sequences also indicated that the mutant but not the wild-type proteins retained the N-terminal methionine, a difference which can presumably be accounted for by the fact that different strains of *E. coli* were used to express the wild-type and mutant proteins.

The proteins were also analysed by electrospray mass spectrometry, the results of which are given in Table 1. Notwithstanding its large mass, results of reasonable precision were obtained for the intact enzyme; the masses calculated from the amino acid sequence are in agreement with the results of N-terminal sequencing, indicating that the N-terminal methionine has been removed from the wild-type enzyme but not from the R47E mutant. In the case of the haem domains, the measured masses were approx. 511 mass units less than those calculated from the sequence, which corresponds to three or four amino acid residues. The results of the N-terminal sequencing indicate there are no residues missing from the N-terminus of either the wild-type or R47E haem domains. At the C-terminus of the haem domain construct, the predicted sequence is ...AKKVR; removal of the last four residues would lead to a loss of 511 mass units. The mass-spectrometry results do not allow us to distinguish between the removal of four residues (KKVR) from the C-terminus and the removal of the N-terminal methionine together with three residues (KVR) from the C-terminus. In any event, it appears that the expressed haem domains have been truncated at the C-terminus, presumably by *E. coli* proteases.

Table 1 Molecular mass measured by electrospray mass spectrometry, for wild-type and R47E mutant cytochrome *P*450 BM3

The calculated masses were obtained from the protein sequence [12] using the software on the mass spectrometer, assuming that all the co-factors, haem, FAD and FMN had been removed.

Protein	Measured mass	Calculated mass	
Intact enzyme			
Wild-type	117 648 ± 74	117 782 ^a	117 651 ^b
R47E mutant	117 756 ± 11	117 755 ^a	117 623 ^b
Haem domain			
Wild-type	53 350 ± 5	53 342 ^c	53 346 ^d
R47E mutant	53 330 ± 13	53 314 ^c	53 317 ^d

^a Mass calculated with initiating methionine still present.

^b Mass calculated with initiating methionine removed.

^c Mass calculated with initiating methionine and three C-terminal residues removed.

^d Mass calculated with initiating methionine still present and four C-terminal residues removed.

Catalytic activity

In the R47E mutant the positively charged arginine side-chain has been replaced by a negatively charged glutamate side-chain. We therefore compared the catalytic activity of the wild-type and mutant enzymes not only with saturated fatty acids, but also with alkyl trimethylammonium compounds; the results are shown in Table 2. For some of the compounds with high K_m values, the precision of the data was limited by the accessible range of substrate concentrations, in turn limited by micelle formation. The results for the action of the wild-type enzyme on fatty acid substrates are in agreement with published data [10,24,25]. As the chain length increases from C_{12} to C_{16} , k_{cat} progressively increases and K_m progressively decreases, so that k_{cat}/K_m increases 300-fold overall; this is in agreement with the order of activity among saturated fatty acids, $C_{15} = C_{16} > C_{14} > C_{17} > C_{13} > C_{18} > C_{12}$, originally reported by Miura and Fulco [7]. The measured k_{cat} for palmitate, 81 s^{-1} , is somewhat greater than that for the C_{20} unsaturated fatty acid arachidonate (53 s^{-1} ; [11]); these are the fastest catalytic rates yet reported for *P*-450 mono-oxygenase reactions.

The activity of the R47E mutant against myristate and palmitate is characterized by a ~ 7 -fold decrease in k_{cat} and a ~ 3 -fold increase in K_m . The behaviour with laurate as a substrate is somewhat different, k_{cat} being essentially unaltered, while there is a much larger increase in K_m . This may be explained by an

altered mode of binding of laurate to the mutant (see below). Notwithstanding this difference, the mutation leads to a decrease in k_{cat}/K_m by a very similar factor, of 13–21, for all three fatty acid substrates.

To ensure that the rates of reaction measured for the fatty acids with the R47E mutant by estimating NADPH oxidation were not affected by uncoupling of electron transfer from substrate hydroxylation, we measured the formation of peroxide and the formation of hydroxylated fatty acid product during the reaction with laurate. For both the wild-type and R47E mutant, less than 10^{-5} mol of peroxide was formed per mol of NADPH consumed. In the ^1H NMR spectrum of the reaction mixture (Figure 1) the terminal methyl resonance of 11-hydroxylaurate appears as a well-resolved doublet at 1.19 p.p.m., while the corresponding resonances of 9- and 10-hydroxylaurate appear as two closely spaced triplets at ~ 0.9 p.p.m. [16]. The spectra in Figure 1 demonstrate the formation of 9-, 10- and 11-hydroxylaurate in approximately the same ratios by both wild-type and mutant enzyme. It is thus clear that the R47E mutant of *P*-450 BM3 is able to hydroxylate saturated fatty acids effectively.

The three alkyl trimethylammonium compounds, dodecyl- (C_{12} TMA), tetradecyl- (C_{14} TMA) and hexadecyl-trimethylammonium bromide (C_{16} TMA), are relatively poor substrates for the wild-type enzyme, with k_{cat} values only 1–6 % of those of the fatty acids, and K_m values 3- to 13-fold higher, leading to k_{cat}/K_m values 100- to 300-fold lower. (These comparisons have been made between, for example, myristate and C_{14} TMA; as discussed below, in terms of the active-site geometry this may not be an exact comparison.) As would be expected, these positively charged compounds are much better substrates for the R47E mutant enzyme; in the case of C_{14} TMA, for example, k_{cat}/K_m is 26-fold greater with the mutant as compared with the wild-type enzyme. The R47E enzyme shows the same chain-length preference, $C_{16} > C_{14} > C_{12}$, among the alkyl trimethylammonium compounds as does the wild-type enzyme among the fatty acids. C_{12} TMA shows the same somewhat anomalous behaviour as noted above for the fatty acid of the same chain length, with no difference in k_{cat} and a larger difference in K_m between wild-type and R47E enzymes. Overall, k_{cat}/K_m for the alkyl trimethylammonium compounds is 11- to 26-fold greater for the mutant. For both the mutant and particularly the wild-type enzyme, the production of peroxide during the reaction was somewhat greater with C_{12} TMA than with laurate, but was still $< 10^{-3}$ mol of peroxide per mol of NADPH consumed. ^1H NMR spectroscopy of the reaction mixture (Figure 2) showed that both enzymes catalysed hydroxylation of C_{12} TMA at the $\omega-1$, $\omega-2$ and $\omega-3$ positions, in approximately the same ratios.

Table 2 Kinetic constants for the action of wild-type and R47E cytochrome *P*450 BM3 on a series of saturated fatty acids and alkyl trimethylammonium compounds

Enzyme substrate	Wild-type			R47E mutant			k_{cat}/K_m ratio (wild-type/mutant)
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\text{M}^{-1} \cdot \text{s}^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\text{M}^{-1} \cdot \text{s}^{-1}$)	
Fatty acids							
Laurate	26 ± 3	136 ± 4	1.9×10^5	29 ± 6	2000 ± 440	1.5×10^4	12.6
Myristate	55 ± 5	7 ± 2	8.2×10^6	7.0 ± 0.2	18 ± 8	3.9×10^5	21.0
Palmitate	81 ± 17	1.4 ± 0.1	6.0×10^7	12 ± 5	4 ± 3	3.0×10^6	20.0
Trimethylammonium compounds							
C_{12} TMA	1.6 ± 0.3	782 ± 125	2.0×10^3	2.2 ± 0.6	95 ± 14	2.3×10^4	0.087
C_{14} TMA	2.2 ± 0.4	87 ± 34	2.5×10^4	15.1 ± 0.8	23 ± 3	6.6×10^5	0.038
C_{16} TMA	1.0 ± 0.1	5.0 ± 0.6	2.0×10^5	20 ± 3	9 ± 4	2.2×10^6	0.091

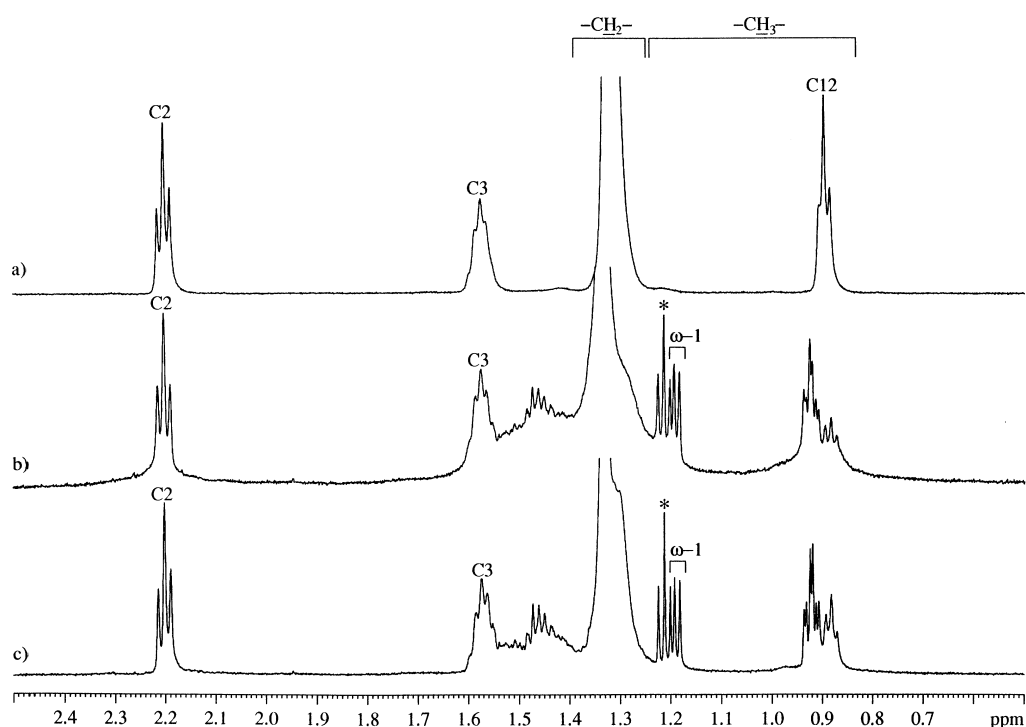


Figure 1 600 MHz ^1H NMR spectra of the reaction mixtures from an incubation of laurate with (b) wild-type and (c) R47E mutant *P*-450 BM3

The spectrum of laurate (a) is included as a reference. The reaction mixtures ($10\ \mu\text{M}$ enzyme, 2.5 mM laurate, 2.5 mM NADPH in 0.1 M phosphate buffer, pH 8.0) were incubated for 24 h at room temperature, followed by 3 days at $4\ ^\circ\text{C}$ to ensure that the reaction had gone to completion. The doublet from the methyl resonance of the product hydroxylated at the $\omega-1$ position is marked. The asterisk indicates a triplet resonance from NADPH.

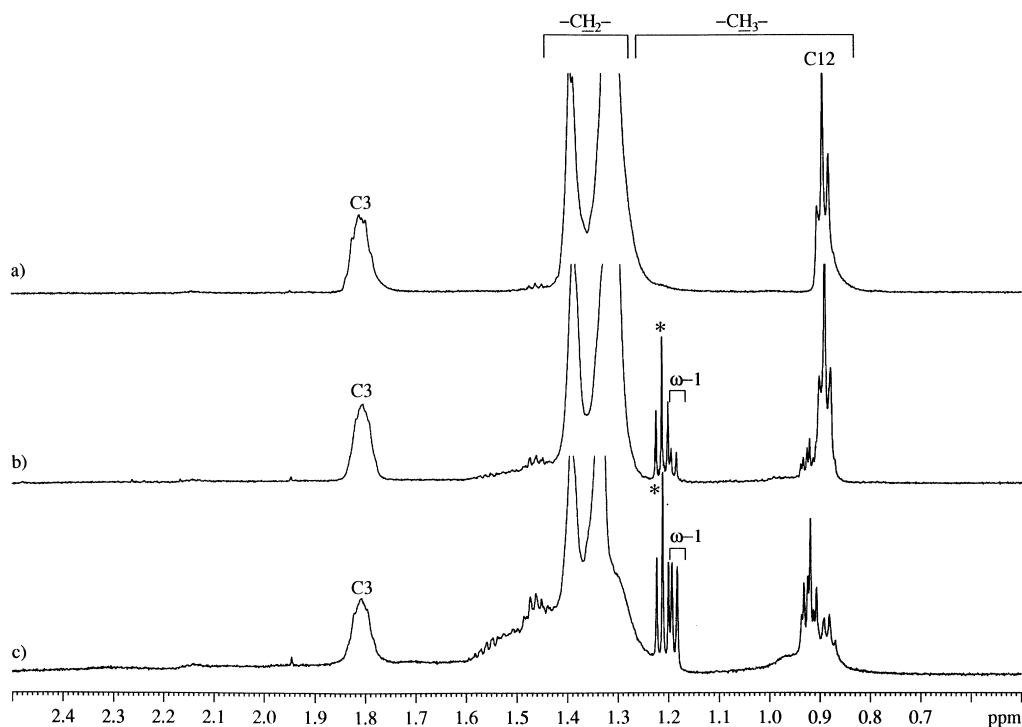


Figure 2 600 MHz ^1H NMR spectra of the reaction mixtures from incubation of C_{12}TMA with (b) wild-type and (c) R47E mutant *P*-450 BM3

The spectrum of C_{12}TMA (a) is included as a reference. The reaction mixtures ($10\ \mu\text{M}$ enzyme, 2.5 mM C_{12}TMA , 2.5 mM NADPH in 0.1 M phosphate buffer, pH 8.0) were incubated for 24 h at room temperature, followed by 3 days at $4\ ^\circ\text{C}$ to ensure that the reaction had gone to completion. The doublet from the methyl resonance of the product hydroxylated at the $\omega-1$ position is marked. The asterisk indicates a triplet resonance from NADPH.

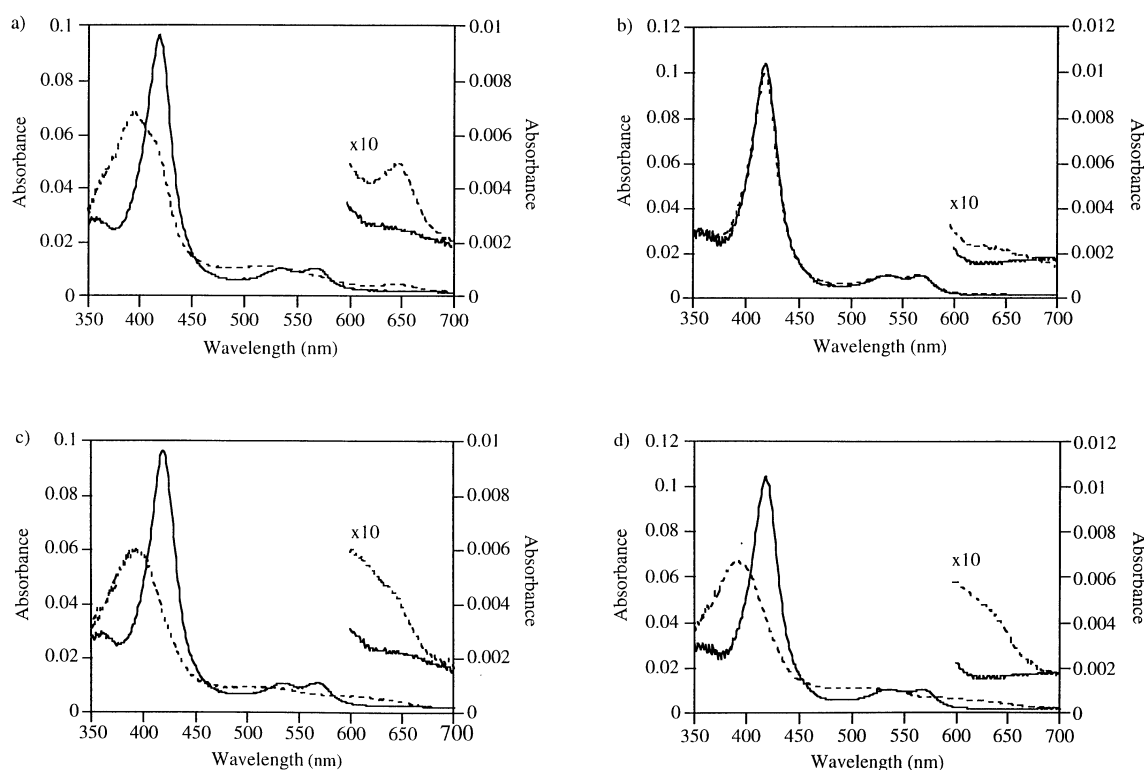


Figure 3 Absorption spectra of haem domains

The absorption spectra of (a, c) the wild-type enzyme (1.22 μM) and (b, d) the R47E mutant enzyme (1.34 μM) in the presence (---) and absence (—) of palmitate (a, b) and C_{16}TMA (c, d).

Optical studies of substrate binding

The purified wild-type and R47E mutant proteins showed electronic absorption spectra typical of cytochromes *P*-450: for the oxidized protein (shown in Figure 3 for the haem domains), a spectrum with well-resolved bands at 570 and 535 nm and a strong Soret band at 418 nm, while for the CO complex of the reduced enzyme (results not shown), the spectrum shows peaks at 547 nm and 448 nm. A shoulder present in the spectra of intact *P*-450 BM3 between 450 nm and 470 nm is indicative of the presence of the flavins [26].

Figure 3 shows the absorption spectra of the haem domains of the wild-type and R47E mutant enzyme in the presence and absence of palmitate and C_{16}TMA . On addition of either substrate to the wild-type protein, a typical 'type I' spectrum is produced, characterized by a decrease in the Soret band at 418 nm with a simultaneous increase in the absorbance at 390 nm and 650 nm (Figures 3a and 3c). On C_{16}TMA binding, the peak at 650 nm is clearly broader than for palmitate binding, perhaps due to micelle formation at the concentration of C_{16}TMA used. This comparison shows that the natural fatty acid substrate and the trimethylammonium compound bind to the wild-type enzyme in a very similar way. In the case of the R47E mutant, binding of C_{16}TMA leads to changes in the optical spectrum essentially identical with those seen with the wild-type enzyme (Figure 3d). The concentration of palmitate which could be used was limited (by micelle formation) to 8 μM , and this was not sufficient to produce any clear change in the absorption spectrum of the haem domain of the R47E mutant (Figure 3b). The difference in K_m for palmitate between wild-type and mutant enzyme is only about a factor of two (Table 2); the lack of effect of 8 μM palmitate on

the absorption spectrum suggests that the difference in K_d for binding to the haem domain must be rather greater than this.

The equilibrium dissociation constants, K_d , for the binding of fatty acids to the wild-type enzyme and of trimethylammonium compounds to the R47E mutant were calculated from the decrease in absorbance at 418 nm on addition of substrate, and are given in Table 3. The affinity of the R47E mutant for the alkyl trimethylammonium compounds is broadly similar (within a factor of less than 3) to that of the wild-type enzyme for fatty acids of comparable chain length, the affinity increasing with increasing chain length.

The binding of the C_{12} substrates

As noted above, the effect of the mutation on the kinetic behaviour of the C_{12} substrates appeared to be anomalous, and the binding of these compounds was therefore studied in more detail. The binding of laurate to the R47E mutant and of C_{12}TMA to the wild-type enzyme was too weak to be determined directly by the changes in the optical spectrum, since the K_d values were comparable with the critical micelle concentrations of these substrates. Approximate K_d estimates of 4.2 mM for the laurate–R47E interaction and 2.2 mM for the C_{12}TMA –wild-type interaction with the haem domains were obtained by competition with the corresponding C_{14} compounds (Table 3). An estimate of $K_d = 10$ mM for the binding of laurate to the haem domain of the R47E mutant obtained from the NMR relaxation experiments described below, was in reasonable agreement with that obtained by competition. For the C_{12} compounds, therefore, the binding to the resting, ferric, state of the enzyme of

Table 3 Equilibrium dissociation constants for substrate binding to wild-type and R47E mutant cytochrome P450 BM3

* Methodology: NMR, determined from the concentration-dependence of the spin-lattice relaxation rate of substrate protons; Optical, determined from the concentration-dependence of the absorbance at 418 nm; Optical competition, determined from the effect of the C₁₂ compound on the binding of a reference compound, the latter determined optically. n.d., not determined.

Enzyme/substrate	Method*	K_d (M)		
		Intact enzyme	Haem domain (ferric state)	Haem domain (ferrous state)
R47E mutant				
C ₁₂ fatty acids	NMR	n.d.	$1.0 (\pm 0.3) \times 10^{-2}$	$3.6 (\pm 0.3) \times 10^{-3}$
C ₁₂ fatty acids	Optical competition	n.d.	$4.2 (\pm 2) \times 10^{-3}$	n.d.
Wild-type				
C ₁₂ fatty acids	NMR	n.d.	$8.4 (\pm 0.4) \times 10^{-4}$	$7.9 (\pm 1.6) \times 10^{-5}$
C ₁₂ fatty acids	Optical	$2.7 (\pm 0.5) \times 10^{-4}$	$6.3 (\pm 1) \times 10^{-4}$	n.d.
C ₁₄ fatty acids	Optical	$2.3 (\pm 0.6) \times 10^{-5}$	$2.8 (\pm 0.4) \times 10^{-5}$	n.d.
C ₁₆ fatty acids	Optical	$5.0 (\pm 1.4) \times 10^{-6}$	$8 (\pm 3) \times 10^{-6}$	n.d.
R47E mutant				
C ₁₂ TMA	Optical	$1.8 (\pm 0.3) \times 10^{-4}$	$2.5 (\pm 0.5) \times 10^{-4}$	n.d.
C ₁₄ TMA	Optical	$4.1 (\pm 0.7) \times 10^{-5}$	$5.2 (\pm 0.5) \times 10^{-5}$	n.d.
C ₁₆ TMA	Optical	$4.0 (\pm 1.5) \times 10^{-6}$	$1.1 (\pm 0.3) \times 10^{-5}$	n.d.
Wild-type				
C ₁₂ TMA	Optical competition	n.d.	$2.3 (\pm 1) \times 10^{-3}$	n.d.

Table 4 Paramagnetic relaxation times and iron–proton distances for laurate and 12-bromolaurate binding to the haem domain of wild-type and R47E mutant cytochrome P-450 BM3

Relaxation measurements on the ferric state were carried out at 600 MHz and those on the ferrous state at 250 MHz. —, resonance not resolved.

Protein	Substrate	Parameter	C ₁₀ -CH ₂ -	C ₁₁ -CH ₂ -	C ₁₂ -CH ₂ X ^a
Ferric					
R47E mutant	Laurate	T_{1M} (ms)	—	—	0.43 ± 0.03
		r (Å)	—	—	6.05 ± 0.1
Wild-type ^b		r (Å)	—	—	7.6 ± 0.3
Ferrous					
R47E mutant	Laurate	T_{1M} (ms)	—	—	5.3 ± 0.2
		r (Å)	—	—	4.89 ± 0.02
Wild-type ^b		r (Å)	—	—	5.1 ± 0.2
R47E mutant	12-Bromolaurate	T_{1M} (ms)	0.17 ± 0.01	0.26 ± 0.01	5.1 ± 0.1
		r (Å)	2.75 ± 0.03	2.95 ± 0.02	4.85 ± 0.02
Wild-type ^b		r (Å)	3.0 ± 0.1	3.1 ± 0.1	5.1 ± 0.1

^a X = H or Br.

^b Data from [13,15].

a substrate having the same charge as residue 47 is roughly ten times weaker than that of a substrate having the opposite charge (and thus able to make an ion-pair interaction with this residue).

The paramagnetic relaxation effects of the haem iron on the protons of the substrates laurate and 12-bromolaurate were measured and used to obtain estimates of the iron–proton distances as described in detail previously [13]. The relaxation times and the derived distances are given in Table 4, where they are compared with the distances for the wild-type enzyme reported previously [13,15]. In the initial complex with the ferric enzyme, the protons on the terminal methyl group of laurate are seen to be significantly closer to the iron in the mutant than in the wild-type haem domain (6.05 Å versus 7.60 Å). On reduction of the complex to the ferrous state, there is a structural change which leads to a substantial movement of the substrate towards

the haem, into position for hydroxylation [15], and this is also seen in the mutant enzyme. In the ferrous complex, the difference in position of the laurate methyl group is much smaller (approx. 0.2 Å) and only marginally significant. Similar observations were made with 12-bromolaurate, a good substrate which has a better-resolved NMR spectrum, thus allowing the positions of the C₁₀ and C₁₁ methylene groups (the sites of hydroxylation) to be determined; like the methyl group, these are in a very similar position in the ferrous complexes of the mutant and wild-type haem domains.

The relaxation experiments also provide estimates of K_d for laurate binding to the haem domain, and these are included in Table 3. In the ferric state, laurate binds weakly to both wild-type and R47E haem domains, about 12-fold more weakly to the latter. As noted previously [15], substrate binding to the ferrous state is significantly stronger than to the ferric state, and comparable with the kinetically determined K_m value. In the ferrous state, laurate binds 45-fold more weakly to the R47E mutant than to the wild-type, a significantly larger difference than that seen in the ferric state.

DISCUSSION

In terms of the specificity constant, k_{cat}/K_m , the replacement of arginine-47 by glutamate in cytochrome P-450 BM3 leads to a clear switch in specificity of the enzyme. Comparing the values for the C₁₄ and C₁₆ substrates, the wild-type enzyme is 300-fold more active with the fatty acid than with the trimethylammonium compound, while the R47E mutant shows very similar activity against both, having a k_{cat} as high as 19 s⁻¹ for hydroxylation of C₁₆TMA. The change in charge state of residue 47 has essentially reciprocal effects on the activity of the enzyme towards the two different, oppositely charged groups of substrates. For the fatty acids, the k_{cat}/K_m values of the wild-type enzyme are 14- to 21-fold higher than those of the mutant, while for the alkyl trimethylammonium compounds the k_{cat}/K_m values are 12- to 26-fold higher for the mutant than for the wild-type. Similarly, the binding constants for the fatty acids to the wild-type enzyme are generally closely comparable with those for the trimethyl-

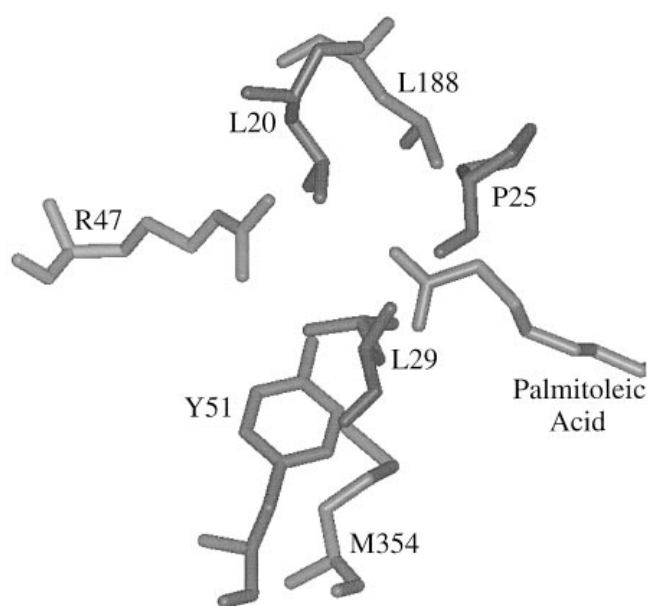


Figure 4 The environment of the substrate carboxylate group in the crystal structure [14] of the complex of palmitoleic acid with the ferric state of the haem domain of *P*-450 BM3

ammonium compounds to the R47E mutant. Exact comparisons are difficult to make in view of the differences in length between arginine and glutamate side-chains, and between fatty acids and alkyl trimethylammonium compounds. A glutamate side-chain is two bonds (approx. 2.3 Å) shorter than an arginine, although the difference in the effective positions of the charges will be slightly less than this. At the same time, recalling that the charge of a trimethylammonium group is very largely delocalized over the N-methyl groups, a C_{14} alkyl trimethylammonium compound is effectively one bond longer than a C_{14} fatty acid.

The R47E mutant is clearly still a reasonably effective fatty acid hydroxylase, forming the same products as the wild-type enzyme in essentially the same ratios, at maximum rates less than an order of magnitude slower. This is in marked contrast with the recent report [17] that this mutant is catalytically inactive towards arachidonic acid and eicosapentaenoic acid and their methyl esters. Since arachidonic acid is epoxidized by *P*-450 BM3 at the 14,15 ($\omega-5$, $\omega-6$) positions [11], it must be able to bind differently in the active site from the saturated fatty acids which are hydroxylated at the $\omega-1$, $\omega-2$ and $\omega-3$ positions. Nonetheless, it is surprising that a difference in binding near the haem should lead to such a difference in the importance of the arginine-47 side-chain, some 15 Å away.

The differences in catalytic activity between the wild-type and mutant enzymes towards the two classes of substrate are smaller than would be expected for the conversion of a significant electrostatic attraction between the substrate and the enzyme into an electrostatic repulsion. In terms of binding, direct comparisons can only be made for the C_{12} compounds, where the affinity for a substrate having the same charge as residue 47 is only approximately ten times weaker than that for a substrate having the opposite charge. These observations, which suggest that arginine-47 does not make a major contribution to substrate binding, can be understood in the light of the very recently published crystal structure of the complex of the ferric state of the enzyme with palmitoleic acid [14]. Figure 4 shows the

environment of the carboxylate group of palmitoleic acid bound to cytochrome *P*-450 BM3. The side-chain of arginine-47 appears to be very flexible even in the complex, but is positioned so as to be able to form an ion-pair with the carboxylate. In addition, the carboxylate forms a second hydrogen bond to the phenolic hydroxy group of tyrosine-51. Assuming that the two classes of substrates bind similarly to the wild-type and mutant enzymes (see below), this structure indicates two possible reasons for the modest effect of a mis-match of charge between residue 47 and the substrate. First, the flexibility of this residue will allow, for example, the side-chain of glutamate-47 to move away from the carboxylate of a fatty acid. At the same time, tyrosine-51 will continue to provide a favourable interaction. Its hydroxy group will act as a hydrogen-bond donor to the carboxylate anion of fatty acid substrates, and will also be able to contribute a favourable interaction between its electronegative oxygen and the cation of trimethylammonium substrates.

It is clear that both the initial binding of the substrate [13,14] and subsequent steps in the catalytic cycle [15,16] involve structural changes in the enzyme. The changes in the optical absorption spectra of the haem on substrate binding to cytochromes *P*-450 have been attributed [27,28] to a change in spin state of the haem iron from low spin ($S = 1/2$) to high spin ($S = 5/2$) accompanying the expulsion of the water molecule from the sixth co-ordination position of the iron. In the case of *P*-450 BM3, the expulsion of the co-ordinated water must result from the structural change accompanying substrate binding, since the substrate binds to the ferric form of the enzyme at some distance from the iron ([13,14]; see also Table 4) and cannot displace the water directly. Where they can be measured, the changes in the optical absorption spectra of the haem on the binding of fatty acids and alkyl trimethylammonium compounds to wild-type and R47E mutant enzyme are comparable, indicating that a similar structural change is induced by substrate binding in each case. The next step in the catalytic cycle, the reduction of the enzyme-substrate complex, is accompanied by a marked movement of the bound substrate towards the iron, into position for hydroxylation [15]; a very similar movement of laurate occurs on reduction of its complex with the haem domain of the R47E mutant, again emphasising the similarity in behaviour of the mutant and wild-type enzymes.

The R47E mutation produces an increase in K_m for fatty acids and a decrease in K_m for alkyl trimethylammonium compounds which, though quite small in all cases, is noticeably less for the C_{14} and C_{16} compounds (< 4 -fold) than for the C_{12} compounds (13- to 15-fold). One would expect a proportionately larger contribution of the electrostatic interaction to the overall binding affinity of compounds with shorter alkyl chains, and hence weaker hydrophobic interactions. It is known that laurate is the shortest-chain fatty acid which is a substrate for *P*-450 BM3 [7], and its alkyl chain is only just long enough to extend from arginine-47 to the position close to the haem required for hydroxylation at the $\omega-1$, $\omega-2$, and $\omega-3$ positions (see [15]). It is thus possible that the mode of binding of laurate to the wild-type enzyme is a balance between optimizing the interactions of the carboxylate with arginine-47 and tyrosine-51 and those of the alkyl chain with the hydrophobic remainder of the binding site. When the interaction with the arginine residue is abolished (indeed made repulsive) in the R47E mutant, the optimum hydrophobic interactions may lead to a different mode of binding. Support for this comes from the paramagnetic relaxation experiments, which show that laurate does bind differently to the R47E mutant than to the wild-type enzyme in the ferric form of the complex, the methyl group of laurate being 1.5 Å closer to the iron in the mutant. With the longer alkyl chains of palmitate and

myristate, it may be possible to simultaneously optimize both the electrostatic and the hydrophobic interactions, so that little or no change in the mode of binding takes place on the replacement of arginine-47. Similar arguments apply to the binding of the alkyl trimethylammonium compounds to the R47E mutant.

The results reported here thus show that arginine-47 does play a role in the binding of the carboxylate group of fatty acid substrates to cytochrome *P*-450 BM3, though this is not quantitatively a dominant one. This is in good agreement with the conclusions drawn very recently from the crystal structure of an enzyme-substrate complex [14]. They also demonstrate that simple amino acid substitutions can significantly alter the substrate specificity of the enzyme. Taken together with the recent demonstration that the regiospecificity of catalysis can also be altered by simple replacements of phenylalanine-87 [16,17], this suggests that it will be possible to tailor the catalytic activity of this cytochrome *P*-450 for applications in organic synthesis.

We are grateful to Dr. John Miles for providing the expression systems for cytochrome *P*-450 BM3 and its haem domain, to Gavain Sweetman and John Lamb for assistance with mass spectrometry, and to GlaxoWellcome and the Medical Research Council for financial support.

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