Rational re-design of the substrate binding site of flavocytochrome P450 BM3

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Abstract ***Bacillus megaterium*** **P450 BM3 is a fatty acid hydroxylase with selectivity for long chain substrates (C_{12}–C_{20}). Binding or activity with substrates of chain length $< C_{12}$ has not been reported. Rational mutagenesis was used to re-design the enzyme to encourage binding of short chain fatty acids (C_4–C_{10}). We show that wild-type P450 BM3 has activity and weak affinity for substrates as short as butyrate (C_4). However, turnover/binding of short chain substrates is dramatically increased by introducing a novel substrate carboxylate binding site close to the heme. Mutant L181K shows catalytic efficiency (k_{cat}/K_M) increased >13-fold with butyrate, while the L75T/L181K double mutant has k_{cat}/K_M increased >15-fold with hexanoate and binding (K_d) improved >28-fold for butyrate. Removing the arginine 47/lysine 51 carboxylate binding motif at the mouth of the active site disfavours binding of all fatty acids, indicating its importance in the initial recognition of substrates.**

Key words: Cytochrome P450; Substrate recognition; Rational mutagenesis

1. Introduction

Cytochromes P450 (P450s) are cysteine-ligated haem-containing monooxygenases widespread in nature [1]. They have important metabolic functions (including steroid syntheses and transformations), play roles in detoxification of drugs and xenobiotics, and catalyse breakdown of unusual compounds as carbon sources for growth [2,3]. Their predominant reaction is the mono-oxygenation of organic molecules by the activation and insertion of an atom from molecular O_2 (Scheme 1). However, other reactions (e.g. dealkylation) have also been reported [4].

$$RH + O_2 + 2H^+ + 2e^- \rightarrow ROH + H_2O \qquad \text{Scheme 1}$$

Analysis of soluble bacterial P450s such as the camphor hydroxylase from *Pseudomonas putida* and the fatty acid hydroxylase from *Bacillus megaterium* have given insights into the structure and function of their membrane-bound eukaryotic counterparts [5]. The X-ray crystal structures of bacterial P450s have allowed various aspects, key to their function, to be investigated by a rational approach. Bacterial structures also have important biotechnological roles as models for the eukaryotic P450s for e.g. design of new drugs, and have great potential to catalyse novel reactions inaccessible to conventional chemistry.

One of the most intensively studied of the bacterial P450s is the flavocytochrome P450 BM3, a high activity fatty acid hydroxylase from *B. megaterium* [6]. P450 BM3 is a natural fusion of a P450 to a mammalian-like diflavin NADPH-P450 reductase and is of great importance in understanding structure, function and electron transfer in this enzyme class.

In this study, we have altered substrate selectivity in P450 BM3. The substrate (palmitoleate)-bound P450 BM3 structure reveals several hydrophobic residues within van der Waals contact of the substrate [7]. Using rational site-directed mutagenesis, we have replaced selected neutral residues with positively charged or more hydrophilic residues in attempts to pinpoint substrate binding determinants. We have also attempted to copy the fatty acid carboxylate binding motif (R47/Y51, found at the mouth of the active site) at positions further down the substrate binding channel to encourage binding of shorter chain fatty acids. To date, no research has successfully addressed reasons for the observed substrate selectivity profile of this important flavocytochrome, which only has reported activity for fatty acids of chain length C_{12}–C_{20}. Here we provide evidence that rational mutagenesis can be used to convert P450 BM3 to favour binding of shorter chain (C_4–C_{10}) fatty acids.

2. Materials and methods

All P450 BM3 substrates (arachidonic acid and sodium laurate (C_{12}), decanoate (C_{10}), octanoate (C_8), hexanoate (C_6) and butyrate (C_4)) were from Sigma (St. Louis, MO, USA). Oligonucleotide primers were from PE Biosystems. Restriction and DNA modification enzymes were from New England Biolabs. All other reagents were from Sigma. The arachidonic acid stock solution was 33 mM (approximately 10 mg ml^{-1}) in ethanol. All other substrates were prepared as aqueous solutions in the assay buffer for P450 BM3 reactions (20 mM MOPS, pH 7.4, plus 100 mM KCl). NADPH was prepared as a 20 mM stock in ice-cold degassed assay buffer.

Cloning of P450 BM3 and generation of mutants R47A and Y51F has been described previously [8,9]. Site-directed mutagenesis of flavocytochrome P450 BM3 and its haem (P450) domain was according to Kunkel [10,11]. Oligonucleotides used to construct the indicated mutants were as follows (plasmid nomenclature in parentheses, underlined bases indicate mismatches): L181K (pCM21) – 5′-CATTGCTTCATCCTTTGCACGGACCATAC-3′ L181R (pCM22) – 5′-GCTT-

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CATCCCGTGCACGGAC-3′ L437K (pCM23) – 5′-GGTTTTAACGTTTTAGTTTAATATCC-3′ L437R (pCM24) – 5′-GGTTTTAACGTTCGAGTTTCTTTAATATCC-3′ I263K (pCM61) – 5′-GTTTCGTGTCCCGCTTTTAAGAATGTAAT-3′ L75K (pCM59) – 5′-CGTACAAATTTCTTCGCTTGACTTAAG-3′ L181K/L75T (pCM60) – 5′-CGTACAAATTTAGTCGCTTGACTTAAG-3′

Double mutant R47A/Y51F was prepared by mutagenesis of existing clone pCM25 (R47A) [9] using primer 5′-GACTTGATAAGAAGCGCGTTAC-3′. Triple mutant R47A/Y51F/L181K was generated by digestion of the existing plasmid clones pCM28 (R47A/Y51F) and pCM21 (L181K) with *Spe*I and *Afl*II, followed by purification of the smaller (1.5 kb) fragment from the pCM28 digest and the larger (~6.5 kb) fragment from the pCM21 digest by elution after agarose gel electrophoresis. These fragments were re-ligated to generate clone pCM29 (R47A/Y51F/L181K). All mutations were verified by sequencing the complete gene. Plasmid preparations, transformations and dideoxy DNA sequencing (to verify mutations) were performed by standard methods [12]. *Escherichia coli* strain TG1 was used for the expression of the enzymes [13].

Wild-type and mutant P450s BM3 were expressed under the control of the BM3 promoter system, which is highly active in stationary phase. Transformed *E. coli* cells were grown in Luria–Bertani medium supplemented with 25 μg ml^{-1} carbenicillin. Enzymes were purified following the reported protocol [8]. Pure proteins were concentrated to ~200 μM by ultrafiltration, dialysed against 1 l of storage buffer (50 mM Tris–HCl, 1 mM EDTA, pH 7.4) containing 50% (v/v) glycerol, and stored frozen at −80°C.

Steady-state kinetic assays were performed at 30°C in assay buffer. Substrate oxidation assays were performed as described previously [9], monitoring coupled oxidation of NADPH at 340 nm ($\varepsilon_{340} = 6210\ M^{-1}\ cm^{-1}$). Enzyme concentration was ≤3 μM and substrate (carboxylic acids of differing chain length from butyric acid (C_4) up to arachidonic acid (C_{20})) was added over a range of different concentrations, according to the enzyme affinity. The reaction was initiated by addition of NADPH (200 μM). Initial rate of substrate oxidation was measured over the first minute. Specific enzyme rates were calculated and plotted against substrate concentration, and the data fitted to the Michaelis function to generate k_{cat} and K_M parameters.

Substrate dissociation constants (K_d values) were determined by following the low- to high-spin perturbations of the ferric absorption spectra (absorbance shift from 419 to 390 nm) upon substrate-induced removal of the sixth (water) ligand to the heme iron. Titrations were performed either by addition of small (< 0.5 μl) volumes of alcoholic arachidonic acid using a Hamilton syringe (< 5 μl total), or by mixing aliquots of substrate-free enzyme solution with substrate-saturated aqueous solutions containing enzyme at the identical concentration. By mixing substrate-free and substrate-saturated enzyme solutions in different ratios, the desired substrate concentration is obtained. Spectra were recorded between 800 and 300 nm at each point in the titration. Difference spectra were generated and the maximum absorbance difference calculated. Differences were plotted against substrate concentration and fitted to a rectangular hyperbola to generate the K_d in each case. All data manipulations and non-linear least-squares curve-fitting were performed using Origin (Microcal).

Determinations of P450 integrity and concentration were achieved by generating the ferrous-CO adduct of each enzyme. Reduction was performed by addition of sodium dithionite to the enzyme (~5 μM) in assay buffer, and carbon monoxide gas was bubbled into the solution slowly for 60 s to generate the characteristic P450 complex. In all cases, there was near complete (>95%) conversion to the ferrous-CO adduct at 450 nm ('P450'), indicative of active protein. Negligible amounts of the inactive ('P420') species were observed. P450 concentration was determined from the reduced/CO-bound minus reduced difference spectrum, using $\varepsilon_{450-490} = 91\ mM^{-1}\ cm^{-1}$.

3. Results and discussion

Our mutagenesis strategy was based on examination of the active site of palmitoleate-bound P450. Residues in the central region of the long, hydrophobic channel in van der Waals contact with the substrate and less than 10 Å from the heme were selected as mutagenesis targets (Fig. 1). Since the Arg47, Tyr51 motif at the active site mouth is essential both for binding carboxylate groups of long chain substrates and transition state stabilisation [7,9], we constructed a series of mutants in which the selected hydrophobic amino acids (Leu75, Leu181, Ile263 and Leu437) were altered. These were changed to residues capable of forming hydrogen bonds or salt bridges to the carboxylate group of shorter substrates (C_4–C_{10}). We also examined the effects of removing the R47/Y51 motif, to assess whether short-chain fatty acids were then

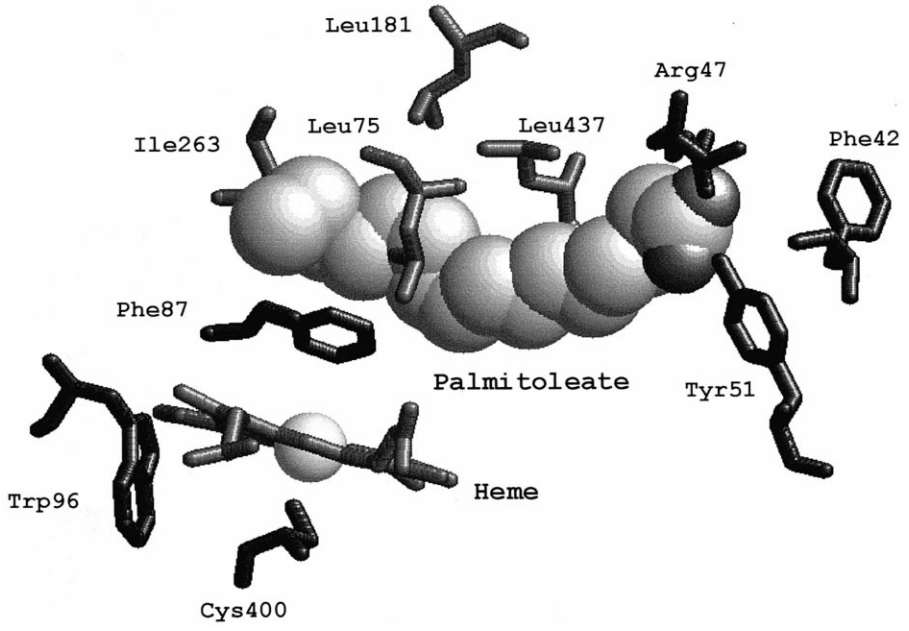

Fig. 1. Important amino acids in the X-ray atomic structure of palmitoleate-bound P450 BM3. Several amino acids have key roles in substrate association (R47, Y51, F42 [9]), regioselectivity of substrate oxidation (F87 [21]), haem incorporation and haem iron spin-state regulation (W96 [22]) and coordination of the haem iron (C400). Other residues within the substrate binding pocket are in van der Waals contact with substrate, and were targets for mutagenesis in this study.

Table 1
Catalytic parameters for wild-type and mutant P450s BM3

Enzyme		Substrate					
		C_4	C_6	C_8	C_{10}	C_{12}	C_{20}
Wild-type	k_{cat} (min^{-1})	88 ± 6	229 ± 26	1364 ± 153	6393 ± 486	5140 ± 90	17100 ± 190
	K_M	387 ± 66 mM	243 ± 49 mM	39 ± 9 mM	6 ± 1 mM	290 ± 20 μM	4.7 ± 0.3 μM
	k_{cat}/K_M (mM min^{-1})	0.23 ± 0.065	0.94 ± 0.35	35 ± 15.5	1066 ± 310	17700 ± 1700	$(3.64 \pm 0.29) \times 10^6$
R47A/Y51F	k_{cat} (min^{-1})	142 ± 16	156 ± 31	692 ± 61	1654 ± 88	2095 ± 88	3234 ± 199
	K_M	463 ± 123 mM	480 ± 134 mM	46 ± 10 mM	4.6 ± 0.6 mM	514 ± 48 μM	33 ± 4 μM
	k_{cat}/K_M (mM min^{-1})	0.307 ± 0.154	0.325 ± 0.215	15.0 ± 5.9	360 ± 75	4080 ± 600	98000 ± 20400
L181K	k_{cat} (min^{-1})	353 ± 7	166 ± 10	747 ± 20	547 ± 35	693 ± 27	3073 ± 174
	K_M	116 ± 8 mM	121 ± 14 mM	4.0 ± 0.3 mM	3.8 ± 0.6 mM	134 ± 20 μM	6.3 ± 0.7 μM
	k_{cat}/K_M (mM min^{-1})	3.04 ± 0.29	1.37 ± 0.27	187 ± 20	144 ± 38	51700 ± 11500	$(4.88 \pm 0.92) \times 10^5$
L181K/L75T	k_{cat} (min^{-1})	205 ± 17	2590 ± 212	3256 ± 128	116 ± 7	269 ± 13	14006 ± 624
	K_M	602 ± 79.5 mM	177 ± 32.5 mM	19.7 ± 2.1 mM	2.67 ± 0.36 mM	316 ± 38 μM	9.3 ± 1.4 μM
	k_{cat}/K_M (mM min^{-1})	0.34 ± 0.08	14.6 ± 4.8	165 ± 27	43 ± 10	850 ± 160	$(1.51 \pm 0.35) \times 10^6$

The k_{cat} and K_M values were determined as described in Section 2. Catalytic efficiency of wild-type and mutant P450s was calculated as the k_{cat}/K_M ratio. ND = 'not determinable', and indicates that catalytic rate was not significantly higher than background, preventing accurate determination of the k_{cat} and K_M values.

capable of diffusing further into the active site without becoming tethered in a position unfavourable for catalysis.

All mutant enzymes had a full complement of flavin and heme, indicating the mutations did not cause major disruption to the tertiary structure. Catalytic (k_{cat} and K_M) and substrate binding (K_d) studies of wild-type P450 BM3 showed there was modest binding (K_M, K_d in mM range) of substrates with chain length shorter than laurate (Tables 1 and 2). However, mutants L181R, L437K, L437R and R47A/Y51F/L181K showed weaker binding and lower catalytic activity than wild-type P450 BM3 with all substrates. The L437K and L437R mutant enzymes were particularly severely affected. L437R had rates of < 10 min^{-1} with all substrates, and maximal activity obtained with L437K was only 120 ± 4 min^{-1} with arachidonate, almost 150-fold lower than wild-type P450 BM3.

Mutants I263K and L75K retained considerable activity (k_{cat}, min^{-1}) with laurate (1014 ± 14 and 1090 ± 34, respectively cf 5140 ± 90 for wild-type P450 BM3) and arachidonate (1443 ± 73 and 1395 ± 54 cf 17100 ± 190 for wild-type P450 BM3), but showed no activity improvement (k_{cat}) with short chain substrates. However, K_M values (mM) for I263K and L75K were lower for hexanoate (49 ± 7.6 and 114 ± 18, respectively, cf 243 ± 49 for wild-type), octanoate (14.6 ± 1.1 and 9.8 ± 1.3 cf 39 ± 9.0) and decanoate (0.85 ± 0.06 and 2.5 ± 0.3 cf 6 ± 1). These K_M improvements lead to small increases in efficiency (k_{cat}/K_M) over wild-type for I263K with hexanoate (4.3-fold) and octanoate (1.9-fold), and for L75K with octanoate (2.7-fold). Spectral measurements also indicated improvements in hexanoate and decanoate binding for L75K (K_d values = 79 ± 19 mM and 0.63 ± 0.09 mM, respectively). A feature of mutant I263K was that, for all substrates, spin-state perturbation on substrate binding was much less than for wild-type P450 BM3. This prevented accurate determination of K_d values for substrates other than laurate and arachidonate. Mutants I263K and L75K showed encouraging changes in substrate chain length specificity. However three other mutants (R47A/Y51F, L181K and L75T/L181K) showed more dramatic alterations in activity and selectivity, and are discussed in more detail below.

3.1. R47A/Y51F

In P450 BM3, both the arginine 47-to-carboxylate salt bridge and the tyrosine 51-to-carboxylate hydrogen bond are important for efficient catalysis [7]. The contribution of R47 is more important to fatty acid binding [9]. By creating the double mutant enzyme R47A/Y51F we sought to investigate two distinct problems: (1) whether total removal of this motif has severe effects on binding of long chain substrates, and (2) whether binding and catalysis of shorter chain substrates is favoured due to their ability to diffuse further into the active site, following removal of a motif that would otherwise 'trap' them distant from the heme iron. Significant decreases in k_{cat} and increases in K_M for both laurate and arachidonate were measured for R47A/Y51F (Table 1). These resulted in 4.3- and 37.1-fold decreases in efficiency (k_{cat}/K_M) for laurate and arachidonate, respectively, compared to wild-type. The cumulative effect of the two mutations is greater than the effect of either in isolation [9]. Thus, both residues are needed for efficient function of P450 BM3, supporting recent predictions [14].

Characterisation of the properties of the R47A/Y51F enzyme with fatty acids shorter than C_{12} chain length showed that there was no improvement in binding (Table 2) and that

Table 2
Binding (dissociation) constants for wild-type and mutant P450s BM3

Enzyme	Substrate and K_d (mM)					
	C_4	C_6	C_8	C_{10}	C_{12}	C_{20}
Wild-type	261 ± 27	131 ± 10	12 ± 2	2.6 ± 0.4	$(241 \pm 7) \times 10^{-3}$	$(3.6 \pm 0.3) \times 10^{-3}$
R47A/Y51F	ND	ND	50 ± 14	ND	ND	$(5.3 \pm 0.2) \times 10^{-3}$
L181K	91 ± 18	246 ± 32	4 ± 1	$(312 \pm 26) \times 10^{-3}$	$(255 \pm 33) \times 10^{-3}$	$(2 \pm 0.2) \times 10^{-3}$
L181K/L75T	9.3 ± 1.9	18.5 ± 5.4	5.1 ± 0.9	0.99 ± 0.12	$(297 \pm 30) \times 10^{-3}$	$(14 \pm 1.5) \times 10^{-3}$

K_d values were determined by spectral titration as described in Section 2. ND = 'not determinable', and indicates that extent of spectral shift induced by the substrate was not great enough to enable the accurate determination of a K_d value.

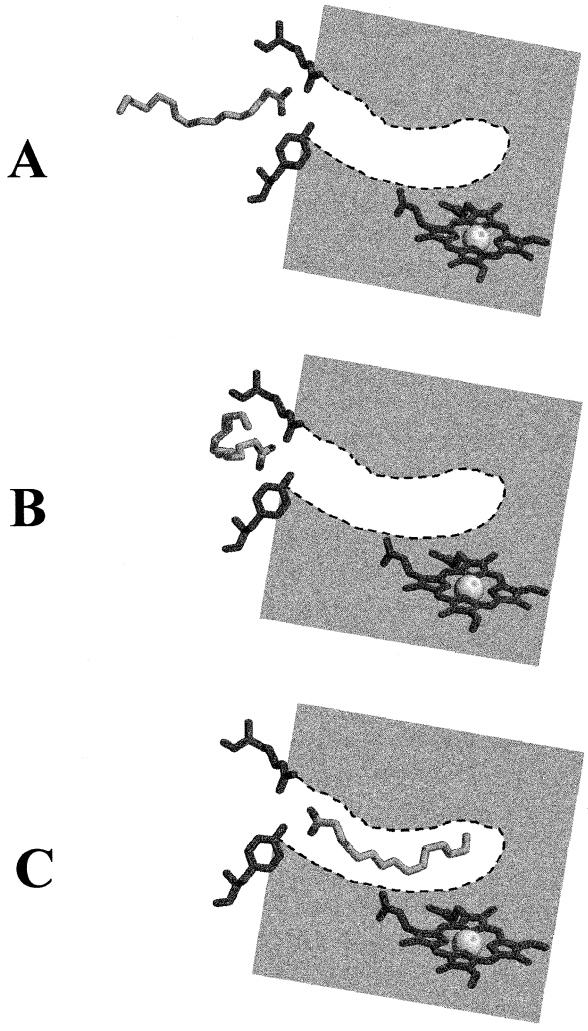

Fig. 2. Model for substrate interaction in P450 BM3. A hydrophobic patch adjacent to the active site entrance makes initial interactions with the alkyl chain of fatty acids. (A) Electrostatic forces orientate the substrate carboxylate group towards R47, and a hydrogen bond between the carboxylate and the hydroxyl group of Y51 further stabilises the binding. (B) The alkyl chain of the substrate is drawn into the active site channel, where favourable hydrophobic interactions ensure strong association. (C) The final catalytically competent orientation is achieved, in readiness for haem reduction and the initiation of the catalytic cycle.

catalytic efficiency was reduced >2-fold for C_6 to C_{10} substrates. For butyrate, there was no significant difference in catalytic efficiency between R47A/Y51F and wild-type (Table 1). Clearly, removal of the motif does not improve the short chain selectivity by preventing molecules from binding at the mouth of the active site. In fact, it appears to be important for the binding of substrates of all chain length, probably by the recognition of substrates from the bulk solution (Fig. 2).

A hydrophobic patch at the surface of the haem domain, adjacent to the active site, is postulated to act as an initial surface for substrate interaction [7]. The palmitic acid binding properties of a P25Q mutant in this region (K_d increased ~100-fold) are consistent with this postulate [15]. However, substrate cannot enter directly into the long hydrophobic active site channel, since mutual hydrophobic effects cannot be transmitted long-range in solution. Instead, we consider that the fatty acid carboxylate is attracted by electrostatic (solution-transmissible) forces to the guanidinium group of R47 at the mouth of the active site. Once this occurs, a hydrogen bond to the hydroxyl group of Y51 can also form. At this point, there is a strong driving force for the movement of the long hydrophobic tail of the fatty acid from the polar solvent phase into the substrate binding channel. Once inside, interactions with several hydrophobic side-chains occur. Thus, the R47/Y51 motif is critical for guidance of fatty acids into the active site.

3.2. L181K

This was the most successful single mutant studied. L181K has decreased catalytic efficiency with long chain substrates, but markedly improved efficiency with shorter chain fatty acids, particularly butyrate (13-fold over wild-type) and octanoate (5-fold) (Table 1). Catalytic efficiency is not significantly different from wild-type for hexanoate, and is decreased 7-fold for decanoate. However, in both cases it is k_{cat} that is unfavourably affected. The K_M for hexanoate and decanoate is actually decreased compared to wild-type (Table 1). The L181K K_d is also decreased for butyrate, octanoate and decanoate. Mutant L181K shows large conversion in substrate selectivity, with the ratio of catalytic efficiency (k_{cat}/K_M) for short chain fatty acids to that for arachidonate increased 99-fold for butyrate and 40-fold for octanoate over wild-type.

Interestingly, L181R does not show similar characteristics and is severely affected in catalysis. The k_{cat} values for this mutant are <40 min^{-1} for all substrates $\leq C_{10}$, and only 584 ± 14 min^{-1} for arachidonate. Substrates of chain length $< C_{12}$ do not induce a significant spin-state shift in the L181R heme, preventing determination of K_d. Clearly, length and/or mobility of the lysine sidechain are critical factor(s) in promoting the observed substrate selectivity changes in L181K. Also notable is that triple mutant R47A/Y51F/L181K is severely impaired in binding and catalysis with all substrates. There is no measurable activity with substrates of chain length $< C_{10}$, and activity is only 1213 ± 38 min^{-1} with arachidonate. Clearly the L181K mutation cannot compensate for the absence of the R47/Y51 substrate binding motif.

3.3. L75T/L181K

In this double mutant, we combined the best single mutation (L181K) with a second mutation in which L75 was mutated to an amino acid (threonine) with a hydroxyl containing side-chain. Here, we attempted to recreate, close to the heme, a motif (lysine/threonine) with similar chemical character and bonding capability to the R47/Y51 motif at the active site mouth. The L75T/L181K double mutant also showed marked alterations in substrate selectivity (Tables 1 and 2). The most striking improvements in catalytic properties of L75T/L181K are the large increases in k_{cat} with hexanoate and octanoate. K_M values for these substrates are also lower than for wild-type, leading to 15.5-fold (hexanoate) and 4.7-fold (octanoate) increases in catalytic efficiency. K_d measurements also indicate considerable improvements in binding of butyrate and hexanoate, with the K_d for octanoate essentially unaltered from that for L181K. Catalytic efficiency with butyrate is also unaltered compared to L181K. The L75T/L181K double mutant has a binding and catalytic profile distinct from the L181K single mutant, with improved catalytic parameters for hexanoate.

Few amino acids are well conserved throughout the P450s, exceptions being those in the heme binding motif around the absolutely conserved cysteine that provides the sulfhydryl ligand to the heme iron (C400 in P450 BM3) [16]. However, the leucine corresponding to L181 in P450 BM3 is conserved in several P450s, particularly in members of the CYP 4 family – fatty acid hydroxylases. In many P450s, a hydrophobic residue is conserved at this position, usually a phenylalanine in P450s exhibiting specificity for aromatic substrates [17]. One of the most interesting of all P450 protein engineering results was obtained by mutation at the position corresponding to L181 in mouse P450s 2A4 and 2A5. These two enzymes differ by only 11 amino acids, but have marked differences in substrate specificity. P450 2A4 catalyses 15α-hydroxylation of testosterone and has a leucine at position 209 (corresponding to L181 in P450 BM3). P450 2A5 catalyses coumarin 7-hydroxylation and possesses a phenylalanine here. Swapping these residues reverses substrate specificity, such that P450 2A4 L209F catalyses coumarin hydroxylation and vice versa for testosterone [18]. Thus it is possible that L181 of P450 BM3 is located at a position of importance in dictating P450 substrate selectivity. Leucine 181 is located in the centre of the F-helix, a motif postulated to be involved in substrate interactions as part of SRS2 (substrate recognition sequence 2) in Gotoh's model [19]. The leucine is also conserved as the final residue of the steroid recognition 'GERL' motif in P450s of subfamilies 11A and 11B [20].

4. Conclusions

In this study, we have demonstrated for the first time that P450 BM3 has measurable affinity for alkanoic acids shorter than C_{12}, and also that preference for fatty acids of this chain length can be selectively enhanced by rational mutagenesis. The single mutation L181K reduced the K_d for decanoate to micromolar levels (312 ± 26 μM), and induced large increases in catalytic efficiency and affinity (K_d) for butyrate and octanoate. By introducing a second mutation (L75T), some further improvements were obtained. In particular, mutant L75T/L181K showed a huge kinetic improvement with hexanoate. Mutants L181K and L75T/L181K are considerably improved enzymes for the binding and catalysis of short-chain fatty acids, providing evidence for a key role for L181 in controlling P450 BM3 substrate selectivity.

Removal of the carboxylate binding R47/Y51 motif diminishes catalytic capacity with all substrate chain lengths, indicating its importance in preliminary substrate binding interactions (Fig. 2). Clearly, this motif should not be altered in future attempts to re-engineer P450 BM3 fatty acid chain length selectivity.

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