The role of tryptophan 97 of cytochrome *P*450 BM3 from *Bacillus megaterium* in catalytic function

Evidence against the 'covalent switching' hypothesis of *P*-450 electron transfer

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The 'Covalent Switching' hypothesis suggests that a strongly conserved tryptophan residue acts as a mediator of electron-transfer flow between redox partners in cytochrome *P*-450 systems [Baldwin, Morris and Richards (1991) Proc. R. Soc. London B **245**, 43–51]. We have investigated the effect of alteration of the conserved tryptophan (Trp-97) in cytochrome *P*-450 BM3 (*P*-450 102) from *Bacillus megaterium*. Replacement of Trp-97 with Ala, Phe or Tyr results in a decrease in the natural haem content and alters the resting spin state of the remaining haem in the purified mutant enzymes. However, kinetic analyses indicate that the mutant enzymes retain high levels of catalytic activity. C.d. and e.p.r. spectroscopy also reveal little alteration in secondary structure or change in the pattern of haem ligation. These findings cast doubt on the covalent switching mechanism of intermolecular electron flow in the *P*-450s, but indicate that this residue plays a role in the association of the haem prosthetic group.

INTRODUCTION

The cytochrome (cyt)-*P*-450-dependent mono-oxygenase (*P*-450) system is found throughout Nature, from bacteria to man, where it is involved in the oxidation of many organic compounds. One atom of O_2 is inserted into the primary product of the reaction, the second atom being reduced to water by NAD(P)H. The importance of these enzymes in microbial fermentation of unusual carbon sources, mammalian drug metabolism and the disposal and the bioactivation of carcinogens has been well documented (Asperger and Kleber, 1991; Muller et al., 1991; Guengerich, 1991; Crespi et al., 1991; Ishikawa, 1992).

Two distinct classes of *P*-450 systems have been recognized. These differ in the protein components responsible for the transfer of electrons onto cyt *P*-450. Class I systems require three proteins: a flavin-containing reductase, an iron–sulphur redoxin and a haem-containing moiety. Members of this class are typified by the camphor hydroxylase P-450*cam* from *Pseudomonas putida*. The three-dimensional structure of P-450*cam* has been solved at high resolution (Poulos et al., 1986). Knowledge of its structure in conjunction with analysis of the properties of various site-directed mutants of the *P*-450 system and ancillary proteins has shed light on structure–function relationships (Martinis et al., 1991; Poulos and Raag, 1992). Class II *P*-450 systems require an NADPH–cyt *P*-450 reductase that contains both FAD and FMN, and are typified by membrane-bound enzymes from mammalian liver. Cyt *P*-450 BM3 from *Bacillus megaterium* is the only known prokaryotic class II *P*-450 system. This fatty acid mono-oxygenase is a soluble multi-domain enzyme containing the cyt *P*-450 and its redox partner (the cyt *P*-450 reductase) in the same polypeptide chain (Narhi and Fulco, 1986). Thus *P*-450 BM3 provides a simple model of class II enzymes. Recently, it has been shown that nitric oxide synthase has an analogous structure, with a single polypeptide chain carrying both FAD- and FMN-binding sites plus a *P*-450-like haem-containing domain (White and Marletta, 1992). The structure of the haem-containing domain of *P*-450 BM3 has recently been determined (Ravichandran et al., 1993).

One of the unanswered questions concerning the function of the *P*-450 is the route of electron transfer from the ancillary redox protein (iron–sulphur redoxin or flavoprotein reductase) to cyt *P*-450. In the P-450*cam* system, it is well established that the C-terminal portion of putidaredoxin (the iron–sulphur protein) is essential for catalysis (Sligar et al., 1974). More recent studies have identified Trp-106, the C-terminal residue, as essential for catalytic function, since its removal or replacement with a non-aromatic residue abolishes activity. Mutant enzymes with Tyr or Phe substitutions at this site retain 20 and 44% of activity respectively (Davies et al., 1990). It is possible that Trp-106 is critical for the docking of putidaredoxin with P450*cam*. Baldwin et al. (1991) have proposed a role for this tryptophan residue in their 'covalent switching' scheme for electron transfer in the *P*-450 s.

Additional support for the hypothesis comes from comparisons of the amino acid sequences of iron–sulphur protein components from mitochondrial class I systems (Munro et al., 1992). Alignment of the approx. 200 *P*-450 amino acid sequences available (Nebert et al., 1991) reveals several residues conserved throughout both *P*-450 classes. Examples include the cysteine ligand to the haem iron, the residues involved in ionic interactions with haem propionates and a threonine implicated in the interaction of oxygen with the *P*-450 (Nelson and Strobel, 1988; Zvelebil et al., 1991). When class II *P*-450 s are examined separately, a further Trp residue, equivalent to Trp-97 in *P*-450 BM3, is seen to be almost totally conserved. This residue is also present in the mitochondrial class I systems (Baldwin et al., 1991; Munro et al., 1992) and has been suggested to play an equivalent role *intramolecularly* to that played *intermolecularly* by Trp-106 of

Abbreviations used: cyt, cytochrome; ETM, electron-transfer mediator; *P*-450, cyt-*P*-450-linked mono-oxygenase.

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putidaredoxin in the P450*cam* system, i.e. acting as an electron-transfer mediator (ETM). In their 'covalent switching' mechanism, Baldwin et al. (1991) postulate that electron transfer occurs via redistribution of electron pairs through a framework of momentarily fixed nuclei. In polypeptides, transfer may occur with an electron departing from a donor atom bonded to sulphur and arriving on an acceptor atom, linked to another sulphur. Their 'Type A' mechanism requires the presence of an interposed disulphide bond and involves the transient formation of two disulphide linkages. For regions in which sulphur atoms are too distant for this process, their 'Type B' mechanism interposes aromatic amino acids, such as tryptophan, to promote electron transfer. Trp-106 of putidaredoxin is postulated to be the aromatic ETM in a 'Type B' switching mechanism for P-450*cam* (Baldwin et al., 1991). Trp-97 of P-450 BM3 would be expected to perform the same role in the class II system.

In the present paper, we have tested the covalent switching hypothesis by engineering site-directed mutants of *P*-450 BM3: W97F, W97Y and W97A. The mutant enzymes have been purified and characterized by enzymological, spectral and biophysical methods.

EXPERIMENTAL

Escherichia coli strains, and plasmid and bacteriophage vectors

The following *E. coli* strains were used: TG1 (*sup*E, *hsd*Δ5, *thi*, Δ(*lac–pro*AB) F′[*tra*D36, *pro*AB$^+$, *lac*I^q, *lac*ZΔM15]) for propagation of M13 bacteriophage and expression of wild-type and mutant *P*-450 BM3 proteins (Gibson, 1984); MV1190 (*sup*E, *thi*, Δ(*lac–pro*AB) Δ(*srl–rec*A)306::Tn10(tet^r) F′(*tra*D36, *pro*AB$^+$, *lac*I^q, *lac*ZΔM15) as host for pUC118 (Sambrook et al., 1989) and DH1 (*sup*E44, *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *rel*A1) as host for pGLW11 (Hanahan, 1983). Bacteriophage M13mp18 was used to provide templates for DNA sequencing and oligonucleotide-directed mutagenesis (Yanisch-Perron et al., 1985). Plasmids pUC118 (Vieira and Messing, 1987) and pGLW11 (a gift from Dr. I. Hunter, Institute of Genetics, University of Glasgow) were used for the expression of genes encoding wild-type and mutant *P*-450 BM3 proteins.

Oligonucleotide-directed mutagenesis

The method of Zoller and Smith (1983) was used to mutate the *P*-450 BM3 codon for Trp-97 with the following oligonucleotide primers: p30, GCGCTTTTTT**GA**AATTTTTTTC (clone mBM6, W97F); p31, GCGCTTTTTT**AT**AATTTTTTTC (clone mBM7, W97Y); and p32, CGCGTTTTTT**GC**ATTTTTTTC (clone mBM8, W97A). The amino acid substitutions directed and the resultant M13 clones are indicated. Each oligonucleotide primer hybridizes to positions 1841–1820 in the *P*-450 BM3 gene sequence (Ruettinger et al., 1989). Mismatches are indicated by the bases underlined and emboldened. The M13 clone mJM2 was used as a template in the mutagenesis reaction. This clone contains the intact *P*-450 BM3 gene amplified from *B. megaterium* (A.T.C.C. 14581) genomic DNA as previously described (Miles et al., 1992). The mutant strand from the mutagenesis reaction was selected by using phosphorothioates with the Amersham International oligonucleotide-directed mutagenesis kit (Sayers et al., 1988). Each mutant clone was fully sequenced, and only the desired mutations were detected. Mutated genes were sub-cloned as *Eco*RI–*Bam*HI fragments into plasmid vectors pUC118 and pGLW11 for expression purposes. Clones were designated pUCBM 30–32 and pGLWBM 30–32 according to the vector and mutagenic primer used.

Other molecular-biology techniques

DNA manipulations, bacterial transformations, restriction-enzyme digests and other molecular techniques were performed by standard methods (Sambrook et al., 1989).

Expression and purification of wild-type and mutant *P*-450 BM3 proteins

Transformants carrying genes encoding wild-type or mutant *P*-450 BM3s were grown overnight to high cell density in Terrific Broth plus antibiotic (ampicillin) (Tartof and Hobbs, 1987). Isopropyl-β-D-thiogalactopyranoside inducer (final concentration 25 μg/ml) was added to facilitate expression from clones in plasmid vectors pGLW11 and pUC118. Wild-type *P*-450 BM3 was expressed from plasmid pJM23 under the control of its own promoter and without induction, as previously described (Miles et al., 1992).

Wet cell pellets (25–30 g) were the starting points for purification of proteins. Cells were broken open with a French press (6.4 MPa) and proteins precipitating with 20–60%-satd. $(NH_4)_2SO_4$ were retained for further purification. Wild-type and mutant proteins were purified to homogeneity by ion-exchange chromatography using DEAE–Sephacel, followed by affinity chromatography on 2′,5′-ADP–Sepharose as previously described (Miles et al., 1992). Gel filtration using Sephacryl S-300 HR was used as a final purification step as necessary.

Spectroscopy, and protein and enzyme assays

All u.v.–visible spectroscopy was performed on a Shimadzu 2100 spectrophotometer (Shimadzu Corp., Kyoto, Japan). Protein concentrations were determined by the method of Bradford (1976) and the BCA (Bicinchoninic Acid) technique (Smith et al., 1985) with BSA as standard. Cyt *P*-450 concentrations were estimated by the method of Omura and Sato (1964) by using ϵ_{450} 91 $mM^{-1} \cdot cm^{-1}$ for the reduced plus CO adduct. Haem contents of purified proteins were estimated with a haem quantification assay (Paul et al., 1953). Cyt *c* reductase activity was determined by using ϵ_{550} 21 $mM^{-1} \cdot cm^{-1}$ as previously described, except that the buffer system used was 20 mM Mops, pH 7.4, containing 100 mM KCl (Miles et al., 1992). NADPH-dependent fatty acid hydroxylation was measured at 30 °C in the same buffer containing 0.5 mM sodium laurate and 0.2 mM NADPH, ϵ_{340} 6.2 $mM^{-1} \cdot cm^{-1}$. Oxygenated n-dodecanoic acid products were separated by reverse-phase h.p.l.c. using a C_{18} column as previously described (Okita et al., 1991).

E.p.r. spectra were recorded at a temperature of 10 K under conditions previously described (Miles et al., 1992). An X-band spectrometer (Bruker ER 200D, with a Datasystem) fitted with a flow cryostat (Oxford Instruments p.l.c., ESR9) was used for data collection.

C.d. spectra were recorded at 20 °C on a JASCO J-600 spectropolarimeter. Molar ellipticity values ($[\theta]_m$) were calculated by using a value of 112 (calculated from amino acid sequences) for the mean residue weights. Analysis of the secondary-structure content was undertaken by using the CONTIN procedure (Provencher and Glöckner, 1981).

The N-terminal sequences of the purified polypeptides were determined by Edman degradation at the B.B.S.R.C. Protein Sequencing Facility in Aberdeen, U.K.

Materials

Oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer and used without further purification.

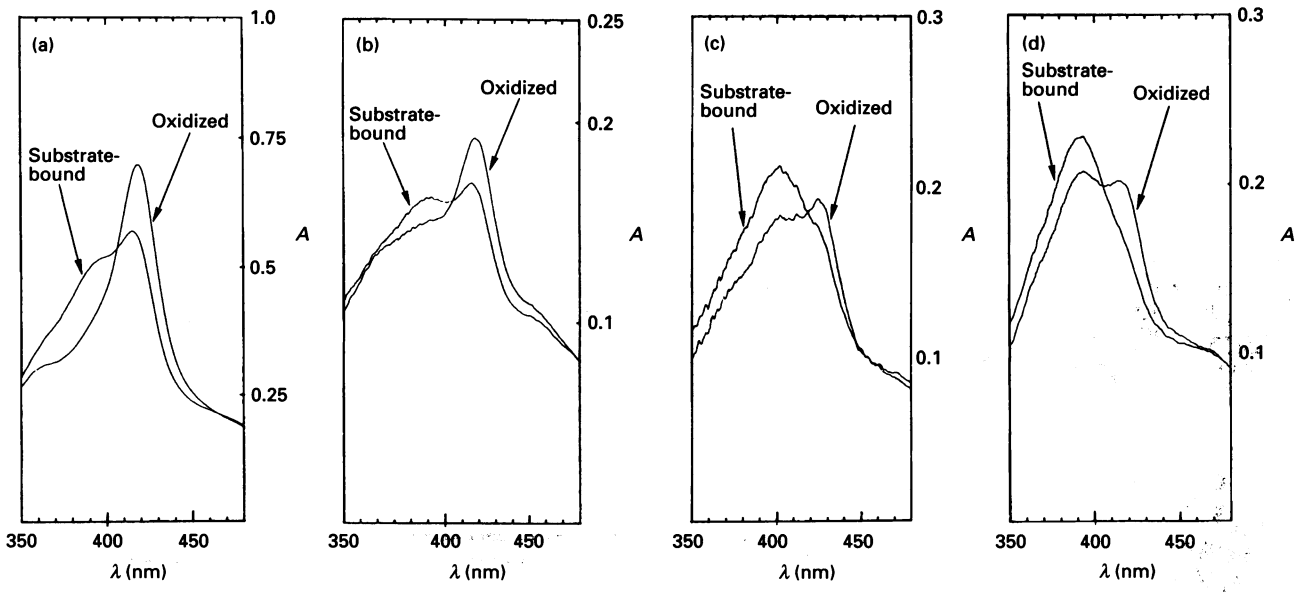

Figure 1 U.v.–visible spectra of oxidized and substrate-bound wild-type *P*-450 BM3 and mutants W97F, W97Y and W97A

Oxidized and substrate-bound (0.5 mM sodium dodecanoate) absorbance spectra (480–350 nm) for the wild-type and mutant enzymes are shown. Proteins were incubated at 15 °C in 20 mM Mops buffer, pH 7.4, containing 100 mM KCl. Mutant polypeptides contain a significantly larger proportion of high-spin haem in the oxidized spectra than native *P*-450 BM3. Spectra: (**a**), wild-type *P*-450 BM3 (4.9 μM); (**b**), mutant W97F (10.4 μM); (**c**), mutant W97Y (4.9 μM); and (**d**), mutant W97A (2 μM).

Molecular-biology reagents were purchased from Boehringer or United States Biochemicals. Radiochemicals were obtained from Amersham International. DEAE–Sephacel was purchased from Pharmacia–LKB. All other reagents were obtained from Sigma.

RESULTS

Purification of wild-type and *P*-450 BM3 mutant and confirmation of desired mutations

Wild-type and mutant enzymes were purified as previously described for the wild-type *P*-450 BM3 (Miles et al., 1992). For most preparations, two successive purification steps using ion-exchange chromatography (DEAE–Sephacel) and affinity chromatography (2′,5′-ADP–Sepharose) proved sufficient for the isolation of pure polypeptides. The mutants, unlike the wild-type enzyme, showed small (< 5%) amounts of material of lower M_r, indicating that limited proteolysis of the former had occurred. A final gel-filtration step (Sephacryl S300 HR) was used for removal of minor contaminants or degradation products as required. Each mutant enzyme has similar electrophoretic mobility to *P*-450 BM3 on SDS/polyacrylamide gels, indicating that all may be isolated in an intact form. Sequencing of the polypeptides by Edman degradation reveals that the N-terminal 15 residues are identical with those predicted from the DNA sequence (Ruettinger et al., 1989). The individually expressed haem domain of *P*-450 BM3 (Miles et al., 1992) has the same N-terminal sequence. Only the desired mutations were detected during DNA sequencing of all of the genes encoding mutants W97F, W97Y and W97A.

Table 1 Protein secondary-structure estimates

Secondary-structure content of wild-type and mutant *P*-450 BM3 systems were predicted by using the method of Provencher and Glöckner (1981) over the absorbance range 240 to 195 nm.

System	α-Helix (%)	β-Sheet (%)	Remainder (%)
P-450 BM3	36±0.36	23±0.64	40±0.77
W97F	37±0.49	24±0.87	39±1.1
W97Y	39±1.6	29±2.4	32±3.7
W97A	38±1.1	25±1.3	37±2.1

U.v.–visible spectra of wild-type and mutant enzymes

For the oxidized enzymes, it is evident from their decreased ratios of A_{419} (haem Soret region) to protein concentration that the mutant enzymes have a lower haem content than does wild-type *P*-450 BM3. This is also apparent from simple inspection of similarly concentrated stocks of the purified proteins. *P*-450 BM3 is clearly red in colour, whereas each of the mutant enzymes appears to be orange. At least three separate purifications of each mutant enzyme were performed and the spectra shown are from the preparations with the greatest haem content. Generally, preparations of mutants W97Y and W97A contain > 20% haem, whereas preparations of mutant W97F contain 10–20% haem.

The visible spectra in the Soret region of the proteins produced from clones pJM23 (wild-type) and pGLWBM30–32 (mutants) are shown in Figure 1. An obvious difference between the mutant and wild-type spectra is that the haem is predominately low-spin in wild-type *P*-450 BM3, whereas the remaining haem in each of the mutants is of mixed spin state. Mutant W97A has a larger content of high-spin haem than the other two mutants under the conditions used (aqueous solution at 15 °C), with the absorbance of the shoulder at ~ 390 nm (indicative of high-spin haem iron) being at least as large as the peak at 419 nm (low-spin). Mutant

W97F has the greatest low-spin content of the mutant polypeptides. In all cases, the high-spin content can be increased by the addition of a fatty acid substrate (Figure 1).

Wild-type and mutant polypeptides all generated normal CO/reduced spectra with absorption maxima at 450 nm following reduction with sodium dithionite and slow bubbling of CO into the cuvette. Measurements of the total absorbance at 450 nm of known amounts of CO-bound forms of the mutant enzymes permitted estimation of relative haem content in the mutant and wild-type enzymes. These provided similar results to those obtained from the haem quantification assay described in the next section. The CO/reduced absorbance ratios A_{450}/A_{280} did not provide a reliable estimate of haem content, since replacement of tryptophan in the mutants leads to large changes in A_{280}.

Determination of haem contents of mutant and wild-type *P*-450 BM3 systems

The quantities of *b*-type haem in preparations of mutant and wild-type *P*-450 BM3 systems were determined by using the haem quantification test (Paul et al., 1953). Three separate determinations were made, and the results obtained differed by $< 4\%$. The results confirmed the sub-stoichiometric haem content in each of the mutant enzymes. The assays indicated that only $\sim$ 20–40% of molecules in all preparations of mutant enzymes contain a molecule of haem. Mutant enzyme used in activity assays exhibited 19 (W97F), 25 (W97Y) and 36% (W97A) haem occupancy. The haem occupancy in the preparation of wild-type P-450 BM3 used was at least 80% (a previous preparation had $\sim$ 90%), whereas that of the independently expressed haem domain of P-450 BM3 was close to 100% (Miles et al., 1992). The cloned P-450 BM3 and its derived haem domain are overexpressed as the major proteins produced in the *E. coli* transformants (Miles et al., 1992; Munro, 1993). As such, small shortfalls in haem content for these wild-type enzymes may reflect difficulty for the cell in matching the rates of haem synthesis and incorporation to the high rate of production of the heterologous polypeptide.

Examination of mutant and wild-type *P*-450 BM3 structure by c.d.

Wild-type and mutant BM3 *P*-450 systems were examined by c.d. in the far-u.v. region (190–260 nm) and in the visible region (320–600 nm) in order to compare their secondary structures and haem environments respectively. The method of Provencher and Glöckner (1981) was used for estimation of secondary structure in wild-type *P*-450 BM3 and mutants W97F, W97Y and W97A. Table 1 shows the relative proportions of α-helix and β-sheet predicted using the CONTIN procedure for data collected between 240 and 195 nm. Overall, α-helical contents are very similar in all cases. In addition, the concentration-corrected spectra obtained for the wild-type and mutant enzymes in the far-u.v. region are virtually superimposable. These data indicate that substitutions of the tryptophan for other aromatic and non-aromatic residues clearly do not cause great perturbation of the secondary structure as determined by c.d. Moreover, although only a sub-population of mutant enzymes contain haem, this appears to have little or no effect on the overall secondary structure. These conclusions are borne out by the results from activity studies in the later section. The protohaem IX moiety of *P*-450 BM3 is non-covalently bound to the enzyme, and it appears that Trp-97 must have an important role in its association. Since no significant structural alterations in the mutant

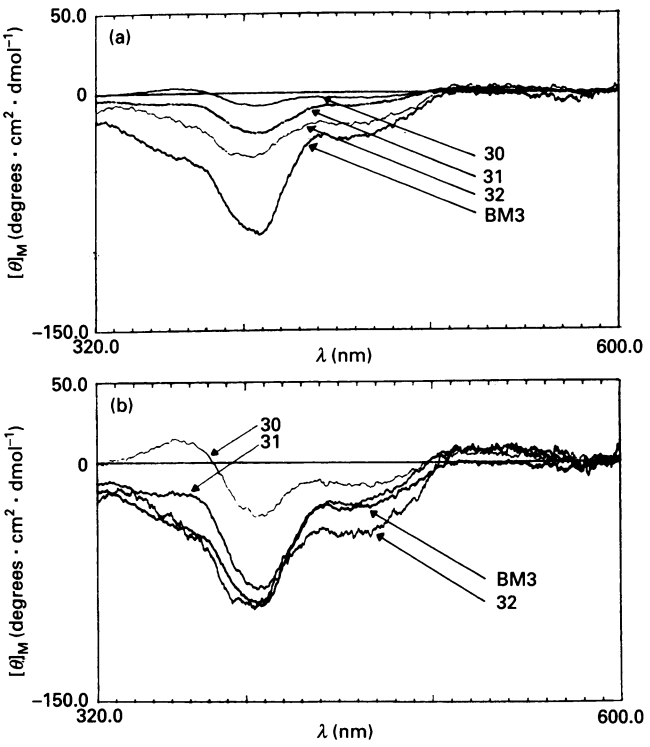

Figure 2 C.d. spectra of wild-type *P*-450 BM3 and mutant enzymes in the visible region

The c.d. spectra of native *P*-450 BM3 and mutants 30 (W97F), 31 (W97Y) and 32 (W97A) in the visible region (λ320–600 nm) are shown. Spectra are corrected for protein concentration such that each trace represents 9.5 μM enzyme (**a**) and for apparent haem content such that each trace represents a haem content equivalent to that of wild-type *P*-450 BM3 (**b**). The spectra were collected as described in the Experimental section.

enzymes can be detected, this role may be conferred by the size or particular aromatic character of the tryptophan residue.

Figure 2(a) shows the spectra of wild-type *P*-450 BM3 and mutants W97F, W97Y and W97A in the visible region (320–600 nm) corrected for the concentration of protein. Clear differences are seen in the intensity of signals collected from the four polypeptides. These differences reflect the varying amounts of haem associated with the wild-type and mutant enzymes. By far the largest signal generated in the Soret region ($\sim$ 410 nm) is that from wild-type *P*-450 BM3, which has the highest haem occupancy. Figure 2(b) shows the same data corrected for estimated haem contents. The 'corrected' spectra exhibit similar features, with shoulders at $\sim$ 370 and $\sim$ 440 nm and a trough centred at 410 nm. The intensity of the signal from mutant W97F (which has the lowest haem content and, hence, the highest correction factor) is somewhat lower than that for wild-type *P*-450 BM3 and the other two enzymes. Mutant W97F also has positive $[\theta]_m$ values in the region approx. 330–390 nm, unlike the other three enzymes. At present, the significance of this phenomenon is uncertain.

E.p.r. studies on *P*-450 BM3 and mutants

The e.p.r. spectra of the three tryptophan mutants (results not shown) indicate the presence of low-spin Fe(III) haem with *g* values which are virtually identical with those obtained previously for the wild-type enzyme and its constituent haem domain (Miles et al., 1992). All five proteins give *g* values of 2.40–2.42, 2.24–2.26

Table 2 Steady-state kinetic values for wild-type and mutant enzymes

Kinetic parameters for the hydroxylation of sodium dodecanoate were measured at 30 °C under conditions previously described (Miles et al., 1992). The K_m and $V_{max.}$ values were estimated by using Lineweaver–Burk and Eadie–Hofstee plots (Fersht, 1984). The 'corrected' $V_{max.}$ for the mutant enzymes is derived by multiplication of their estimated $V_{max.}$ values by a factor equal to the inverse of the apparent proportion of bound haem with respect to that of wild-type *P*-450 BM3 (see the Results section).

Enzyme	K_m (μM)	$V_{max.}$ (s^{-1})	'Corrected' $V_{max.}$ (s^{-1})
P-450 BM3	115	33	–
W97F	125	5	21
W97Y	85	15	46
W97A	110	15	33

and 1.91–1.92. These data indicate clearly that, although the haem content is low, the mutant proteins containing haem are fully active, with haem ligation identical with that of the native protein. This is in agreement with the results of CO difference optical spectroscopy. Thus we can conclude that the haem site remains intact in the tryptophan mutants.

Activity studies on wild-type and mutant enzymes

The steady-state kinetic properties of *P*-450 BM3 and *P*-450 BM30–32 clones were investigated using n-dodecanoic acid ($C_{12:0}$) as substrate. *P*-450 BM3 has optimal activity towards saturated fatty acids of 15- and 16-carbon chain length (Miura and Fulco, 1975). However, $C_{12:0}$ has a higher solubility in aqueous solution than the longer-chain-length substrates. Also, $C_{12:0}$ may be hydroxylated at only a single position when the fatty acid is in limiting concentration (Boddupalli et al., 1990).

The results of the kinetic analyses are shown in Table 2. The wild-type and mutant enzymes exhibit similar affinities towards $C_{12:0}$ as substrate (K_m ~ 120 μM). Interestingly, mutant W97Y appears to have a slightly lower K_m (~ 85–90 μM), indicating a slightly higher affinity for the fatty acid substrate. The $V_{max.}$ for native *P*-450 BM3 is ~ 33 s^{-1} and those for the mutants are somewhat less. However, when the mutant-enzyme $V_{max.}$ values are corrected for haem content (multiplication by the reciprocal of their percentage of wild-type haem content), the values obtained are close to those of the wild-type enzyme. Analysis of fatty acid metabolite formation by h.p.l.c. indicates that wild-type and mutant enzymes all catalyse rapid $C_{12:0}$ mono-oxygenation at rates of at least 85% of those at which NADPH is oxidized. At present, we are uncertain as to whether small differences in catalytic efficiency exist between the wild-type and mutant enzymes, i.e. whether there is slight variation between wild-type and mutant forms in ability to couple NADPH oxidation tightly with fatty acid hydroxylation. Notwithstanding this, it is clear that rapid electron transfer occurs between the reductase and *P*-450 domains of each enzyme. This indicates that Trp-97 is not essential for interdomain electron flow in *P*-450 BM3.

Variation of enzyme concentrations in the presence of saturating levels of substrate indicates that artefactual rate enhancement, by electron transfer between reductase domains of haem-deficient enzyme and normal *P*-450 domains, is not a significant factor. Addition of exogenous reductase domain to mutant-enzyme assays was also found not to cause significant increases in NADPH turnover. All mutant enzymes retain high levels of catalytic activity ('corrected' $V_{max.}$ estimates between ~ 63 and ~ 140% of wild-type $V_{max.}$) and the enzyme with the lowest activity is *P*-450 BM30, which has an aromatic replacement (Trp97Phe). This situation is dissimilar to that observed with Trp-106 mutations of putidaredoxin in P-450*cam*, where non-aromatic replacements lead to large decreases in catalytic $V_{max.}$. Substitution of Trp-97 for alanine in *P*-450 BM3 does not have a similar effect. Thus these findings indicate strongly that Trp-97 is not an aromatic ETM in *P*-450 BM3.

DISCUSSION

Strong evidence is provided in this report that Trp-97 does not have an essential role as an ETM between the flavin and haem domains of *P*-450 BM3. However, replacement of Trp-97 with Ala, Phe and Tyr residues does result in a significant decrease in haem content in the mutant enzymes. E.p.r. analysis demonstrates that mutants containing haem have ligation identical with that in native *P*-450 BM3, although a larger proportion of the haem iron is in its high-spin state in the mutant enzymes compared with wild-type *P*-450 BM3. Alterations in spin state have previously been reported to occur in site-directed mutants of rabbit *P*-450($\omega-1$) (conserved Thr-301 substitutions) and mouse $P\text{-}450_{coh}$ and $P\text{-}450_{15\alpha}$ (substitutions of Cys-209 and Ser-209 respectively) (Imai and Nakamura, 1989; Iwasaki et al., 1991). In all three cases, evidence indicates that the altered residues lie close to the sixth axial ligand to the haem (Iwasaki et al., 1991). This may also be the case for Trp-97 in *P*-450 BM3.

C.d. studies in the far-u.v. region indicate that the amino acid substitutions do not perturb the secondary structure of mutants W97F, W97Y and W97A to any significant extent. Steady-state kinetic experiments show that all of the mutants retain high levels of catalytic activity, when haem content is taken into account. It would appear that Trp-97 may have an important role in haem association/stabilization in *P*-450 BM3. The retention of a similar function for this amino acid might explain its widespread conservation in *P*-450s from higher organisms, particularly class II enzymes. *P*-450 BM3 shares significantly greater amino acid sequence similarity with hepatic *P*-450 IVA1 than with other bacterial *P*-450s (Fulco, 1991) and has been shown to possess a different active-site geometry from the bacterial cytochromes *P*-450*cam* and *P*-450*terp* (Tuck et al., 1992, Ravichandran et al., 1993).

Since both aromatic (Tyr, Phe) and non-aromatic (Ala) replacements for Trp-97 in *P*-450 BM3 result in low levels of haem incorporation, it appears that the aromatic character of tryptophan is not the main determinant of its involvement in haem maintenance. A direct physical contact of Trp-97 with haem is not indicated in the preliminary report of the atomic structure of the haem domain of *P*-450 BM3 (Ravichandran et al., 1993). However, it may be the case that there exists a Trp-97–haem propionate H-bond or perhaps a longer-range interaction that stabilizes the haem moiety. A more precise explanation may be possible when the atomic co-ordinates of the crystallized haem domain of *P*-450 BM3 are available.

At the start of the purification regime for the enzymes, it is apparent that the red colour of *E. coli* transformant cell lysates is much paler in the cases of mutants W97F, W97Y and W97A than for wild-type *P*-450 BM3, indicating that the mutant enzymes are expressed as a mixed population of haem-containing and non-haem-containing molecules. There is no indication of further haem depletion during purification. Thus it would appear that haem deficiency in the mutant enzymes may reflect difficulties in initial insertion of non-covalently bound protohaem IX rather than unstable association of incorporated haem moieties.

Current studies on the haem-deficient mutants are directed towards attempts to titrate exogenous haem into the enzymes. Preliminary studies suggest that this may be possible. We are also investigating the increased proteolytic susceptibility seen with each mutant enzyme compared with wild-type *P*-450 BM3. It is likely that novel sites for host protease enzymes may be exposed in haem-absent sub-populations of mutant enzymes.

The conclusion from the above data regarding the 'Covalent Switching' hypothesis of Baldwin and colleagues is that the conserved tryptophan in *P*-450 BM3 does not act as the ETM which is an essential component of their theory. This, in itself, does not invalidate the hypothesis, but might suggest that an alternative ETM residue exists. Candidates might be Phe-390, Phe-393 or Phe-405, which lie in an apolar region postulated as a redox-partner docking site (Ravichandran et al., 1993). However, the work of Sligar et al. (1991) on the properties of mutants in Trp-106 of putidaredoxin in the P-450*cam* system suggests that decreased catalytic rates with non-aromatic substituted putidaredoxins result from less efficient association with P-450*cam*, and not from the absence of the proposed aromatic ETM. Thus, site-directed mutagenic studies on two different *P*-450 systems have failed to produce evidence in support of the Covalent Switching hypothesis. The purpose of wide cross-species conservation of the tryptophan residue within cyt *P*-450 may be that it serves an important role in the association of haem, as it appears to do in *P*-450 BM3.

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REFERENCES

Asperger, O. and Kleber, H.-P. (1991) in Microbial and Plant Cytochromes P450: Biochemical Characteristics, Genetic Engineering and Practical Implications (Frontiers in Biotransformation, vol. 4) (Ruckpaul, K. and Rein, H., eds.), pp. 1–53, Taylor and Francis, London

Baldwin, J. E., Morris, G. M. and Richards, W. G. (1991) Proc. R. Soc. London B **245**, 43–51

Boddupalli, S. S., Estabrook, R. W. and Peterson, J. A. (1990) J. Biol. Chem. **265**, 4233–4239

Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254

Crespi, C. L., Penman, B. W., Steimel, D. T., Gelboin, H. V. and Gonzalez, F. J. (1991) Carcinogenesis **12**, 355–360

Davies, M. D., Qin, L., Beck, J. L., Suslick, K. S., Koga, H., Horiuchi, T. and Sligar, S. G. (1990) J. Am. Chem. Soc. **112**, 7396–7398

Fersht, A. (1984) Enzyme Structure and Mechanism, 2nd edn., pp. 98–120, W. H. Freeman and Co., New York, NY

Fulco, A. J. (1991) Annu. Rev. Pharmacol. Toxicol. **31**, 177–203

Gibson, T. J. (1984) Ph.D. Thesis, University of Cambridge, Cambridge

Guengerich, F. P. (1991) J. Biol. Chem. **266**, 10019–10022

Hanahan, D. (1983) J. Mol. Biol. **166**, 557–562

Imai, Y. and Nakamura, M. (1989) Biochem. Biophys. Res. Commun. **158**, 717–722

Ishikawa, T. (1992) Trends Biochem. Sci. **19**, 463–468

Iwasaki, I., Juvonen, R., Lindberg, R. and Negishi, M. (1991) J. Biol. Chem. **266**, 3380–3382

Martinis, S. A., Ropp, J. D., Sligar, S. G. and Gunsalus, I. C. (1991) in Microbial and Plant Cytochromes P450: Biochemical Characteristics, Genetic Engineering and Practical Implications (Frontiers in Biotransformation, vol. 4) (Ruckpaul, K. and Rein, H., eds.), pp. 54–86, Taylor and Francis, London

Miles, J. S., Munro, A. W., Rospendowski, B. N., Smith, W. E., McKnight, J. and Thomson, A. J. (1992) Biochem. J. **288**, 503–509

Miura, Y. and Fulco, A. J. (1975) Biochim. Biophys. Acta **388**, 305–317

Muller, H.-G., Schunk, W.-H. and Kargel, E. (1991) in Microbial and Plant Cytochromes P450: Biochemical Characteristics, Genetic Engineering and Practical Implications (Frontiers in Biotransformation, vol. 4) (Ruckpaul, K. and Rein, H., eds.), pp. 87–126, Taylor and Francis, London

Munro, A. W. (1993) Biochem. Soc. Trans. **21**, 316S

Munro, A. W., Malarkey, K. and Miles, J. S. (1992) Biochem. Soc. Trans. **21**, 66S

Narhi, L. O. and Fulco, A. J. (1986) J. Biol. Chem. **261**, 7160–7169

Nebert, D. W., Nelson, D. R., Coon, M. J., Estabrook, R. W., Feyereisen, R., Fujii-Kuriyama, Y., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Loper, J. C., Sato, R., Waterman, M. R. and Waxman, D. J. (1991) DNA Cell Biol. **10**, 1–4

Nelson, D. R. and Strobel, H. W. (1988) J. Biol. Chem. **263**, 6038–6050

Okita, R. T., Clark, J. E., Rice Okita, J. and Masters, B. S. S. (1991) Methods Enzymol. **206**, 432–441

Omura, T. and Sato, R. (1964) J. Biol. Chem. **239**, 2379–2385

Paul, K.-G., Theorell, H. and Akeson, A. (1953) Acta Chem. Scand. **7**, 1284

Poulos, T. L. and Raag, R. (1992) FASEB J. **6**, 674–679

Poulos, T. L., Finzel, B. C. and Howard, A. J. (1986) Biochemistry **25**, 5314–5322

Provencher, S. W. and Glöckner, J. (1981) Biochemistry **20**, 33–37

Ravichandran, K. G., Boddupalli, S. S., Hasemann, C. A., Peterson, J. A. and Deisenhofer, J. (1993) Science **261**, 731–736

Ruettinger, T. T., Wen, L.-P. and Fulco, A. J. (1989) J. Biol. Chem. **264**, 10987–10990

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning, A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Sayers, J. R., Schmidt, W. and Eckstein, F. (1988) Nucleic Acids Res. **16**, 791–802

Sligar, S. G., Debrunner, P. G., Lipscomb, J. D., Namtvedt, M. J. and Gunsalus, I. C. (1974) Proc. Natl. Acad. Sci. U.S.A. **71**, 3906–3910

Sligar, S. G., Filipovic, D. and Stayton, P. S. (1991) Methods Enzymol. **206**, 31–49

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985) Anal. Biochem. **150**, 76–85

Tartof, K. D. and Hobbs, C. A. (1987) Bethesda Res. Lab. Focus **9**, 12

Tuck, S. F., Peterson, J. A. and Ortiz de Montellano, P. R. (1992) J. Biol. Chem. **267**, 5614–5620

Vieira, J. and Messing, J. (1987) Methods Enzymol. **153**, 3–11

White, K. A. and Marletta, M. A. (1992) Biochemistry **31**, 6627–6631

Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene **33**, 103–119

Zoller, M. J. and Smith, M. (1983) Methods Enzymol. **100**, 468–500

Zvelebil, M. J. J. M., Wolf, C. R. and Sternberg, M. J. E. (1991) Protein Eng. **4**, 271–282

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