Flavocytochrome P450 BM3 mutant A264E undergoes substrate-dependent formation of a novel heme iron ligand set

1Hazel M. Girvan, 1Ker R. Marshall, 1Rachel J. Lawson, 1David Leys, 1M. Gordon Joyce, 2John Clarkson, 2W. Ewen Smith, 3Myles R. Cheesman and 1Andrew W. Munro*

1Department of Biochemistry, University of Leicester, The Adrian Building, University Road, Leicester LE1 7RH, UK
2Department of Pure & Applied Chemistry, University of Strathclyde, Thomas Graham Building, 295 Cathedral Street, Glasgow G1 1XL, UK.
3Department of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, UK

Author for correspondence: E mail awm9@le.ac.uk, Phone: 0044 116 252 3464; Fax: 0044 116 252 3369

Running Title: Novel heme ligation in a cytochrome P450
Abbreviations

P450: cytochrome P450 monooxygenase
MCD: magnetic circular dichroism
TMBZ: 3, 3’, 5, 5’-tetramethylbenzidine
SHE: Standard hydrogen electrode
**SUMMARY**

A conserved glutamate covalently attaches the heme to the protein backbone of eukaryotic CYP450s. In the related *Bacillus megaterium* P450 BM3, the corresponding residue is Alanine 264. The A264E mutant was generated and characterized by kinetic and spectroscopic methods. A264E has an altered absorption spectrum from wild-type (Soret maximum at ~ 420.5 nm). Fatty acid substrates produce an inhibitor-like spectral change, with the Soret shifting to 426 nm. Optical titrations with long-chain fatty acids indicate higher affinity for A264E over wild-type. The heme iron midpoint reduction potential in substrate-free A264E is more positive than that for wild-type P450 BM3, and is not changed on substrate binding. EPR, resonance Raman and MCD spectrosopies indicate that A26E remains low spin on substrate binding, unlike wild-type P450 BM3. EPR shows two major species in the substrate-free A264E. The first has normal Cys, aqua iron ligation. The second resembles formate-ligated P450 cam. Saturation with fatty acid increases population of the latter species, suggesting that substrate forces on the glutamate to promote a Cys, Glu ligand set, present in lower amounts in the substrate-free enzyme. A novel charge transfer transition in the near infra-red MCD spectrum provides a spectroscopic signature characteristic of the new A264E heme iron ligation state. A264E retains oxygenase activity, despite glutamate coordination of the iron, indicating that structural rearrangements occur following heme iron reduction to allow dioxygen binding. Glutamate coordination of the heme iron is confirmed by structural studies of the A264E mutant (see Joyce *et al.*, accompanying paper).
INTRODUCTION

The cytochromes P450 (P450s) constitute a superfamily of heme $b$-containing mono-oxygenase enzyme responsible for a huge variety of physiologically and biotechnologically important transformations (e.g., 1, 2). The P450s’ heme iron is ligated to the protein by a conserved cysteinate (the proximal ligand), with a water molecule usually present as the distal axial ligand (3, 4). The P450s are found throughout nature and typically catalyse the reductive scission of dioxygen bound to the heme iron, frequently resulting in hydroxylation of an organic substrate.

Two successive one electron transfers to the P450 are required for oxygenation reactions. The source of the electron are reduced pyridine nucleotides (NADPH or NADH), and electron transfer is usually mediated by one or more redox partner enzymes. In hepatic P450 systems, the redox partner is the diflavin reductase NADPH-cytochrome P450 reductase (CPR), which contains FAD and FMN cofactors (1, 5). In mammalian adrenal systems and many bacterial P450s, electrons are delivered via a two protein redox systems comprising an FAD-containing reductase (adrenodoxin reductase or a ferredoxin reductase) and an iron sulfur protein (ferredoxin) (6, 7). However, other forms of redox systems supporting P450 catalysis are known to exist – including the direct interaction of P450 with hydrogen peroxide to facilitate fatty acid hydroxylation in the Bacillus subtilis P450 BSβ enzyme (8). In recent years, the flavocytochrome P450 BM3 enzyme from Bacillus megaterium has been studied intensively as a “model” P450 enzyme (9). P450 BM3 is a natural, soluble fusion enzyme, in which a fatty acid hydroxylase P450 (N-terminal) is joined to a CPR (C-terminal), creating an efficient electron system capable of hydroxylating a wide range of fatty acids at rates of several thousand events per minute (10, 11). In certain P450s (including P450 BM3), binding of substrate facilitates the first electron transfer from the redox partner, by increasing the reduction potential of the heme iron through removal of the distal water ligand (12, 13). Dioxygen then binds to the ferrous P450 heme iron. Further reduction (by another single electron transfer from the redox partner) accompanied by proton transfer (from solvent, mediated by active site amino acids) facilitates dioxygen cleavage, production of a molecule of $\text{H}_2\text{O}$ and the creation of a highly oxidising ferryl species that ultimately attacks the substrate to facilitate its oxygenation. Dissociation of oxygenated product completes the catalytic cycle (14).
Cytochromes P450 have been isolated from organisms in all domains of life. P450s from thermostable prokaryotes have been studied in order to understand the structural adaptations that facilitate the thermal resistance of the protein and heme system, with a view towards potential biotechnological exploitation (e.g. 15). However, aside from the thermostable isoforms, P450s from mammals and other mesophilic organisms are not particularly stable enzymes. In particular, the heme site is readily denatured by chemical, thermal or pressure treatment (e.g. 16, 17) to generate one or more inactive forms with altered electronic spectral properties - the so-called “P420” species. In P420, the normal ligation of the heme iron is lost, giving rise to a carbon monoxy complex with Soret absorption maximum shifted from close to 450 nm (in the native form) to around 420 nm (in the inactive form). Protonation of the thiolate ligand is thought to underlie this spectral change and concomitant loss of activity (18). Heme can also be dissociated from P450s under harsh conditions, and its reincorporation may not be facile (19). Given the interest in the biotechnological exploitation of P450s in e.g. diagnostics, biosensing and fine chemical synthesis (e.g. 20), there is strong interest in this area. Recent studies have highlighted the power of mutagenesis in the evolution of cytochrome P450 enzymes with altered catalytic properties (21, 22), but a major problem remains their operational stability.

Recent studies on mammalian family 4 (fatty acid hydroxylase) P450 enzymes have revealed unusual stability of binding of the heme in various members of the family. It was shown that SDS-PAGE resolution of the several CYP4 isoforms did not separate heme from the protein, suggesting that there was covalent ligation between the heme macrocycle and the protein (23, 24). In studies of rat liver CYP4A1, a conserved glutamate residue (E318 in this isoform) was found to be the amino acid residue to which the heme had become esterified. The importance of the covalent linkage of heme to the activity of this P450 was demonstrated through mutagenesis of key residues, although the activity in other CYP4A isoforms may not be so sensitive to the extent of covalent ligation of the heme to the protein (24, 25). Studies on mammalian CYP4A1, CYP4A3 and CYP4A11 confirmed that covalent heme linkage to the protein was autocatalytic and occurred due to esterification with the glutamate via the heme 5-methyl group (25). The formation of a porphyrin carbocation species was postulated as an intermediate in the reaction with the conserved glutamate, that facilitates covalent heme ligation (26). Covalent heme linkage has not been recognised in any other P450 isoform, but the ability to stabilise the heme cofactor
may have important ramifications as regards the exploitation of P450s in such areas as biotransformations and toxicological applications.

Flavocytochrome P450 BM3 has been recognised as perhaps the most important P450 enzyme with respect to its capacity to perform biotechnologically-exploitable chemical transformations (9). The wild-type enzyme has been shown to catalyse regio- and stereo-selective hydroxylation and epoxygenation of long chain saturated and unsaturated fatty acids of varying chain length (11, 21), and mutants generated both rationally and by forced evolution have exhibited novel properties such as hydroxylation at the ω-position on fatty acids (rather than the preferred ω-1 to ω-3 positions), and oxygenation of polycyclics, substituted fatty acids, indole, alkanes and short chain fatty acids and alcohols (e.g. 21, 22, 27, 28). However, P450 BM3 exhibits the same structural instability observed in other P450 isoforms as regards the tendency to undergo inactivation at the heme site through P420 formation (16). In this respect, generation of a more stable P450 derivative through covalent attachment of the heme macrocycle is an attractive proposition. The fact that the heme domain of P450 BM3 is strongly related to fatty acid hydroxylases of the CYP4 family suggested that covalent heme linkage might also be feasible in this isoform, and the fact that it has not been observed for the wild-type BM3 could be explained by the presence of an alanine rather than a glutamate residue in the respective position of the conserved I helical region in this P450 (Figure 1) (29).

FIGURE 1 HERE

In this study, we have generated and characterized the A264E variant of P450 BM3 (in both the full length flavocytochrome and the heme domain) and determined the effect of the mutation on the catalytic, spectroscopic, thermodynamic and structural properties of the enzyme. In contrast to the results with the mammalian P450 isoforms, the introduction of a glutamate residue does not result in turnover-dependent covalent linkage of the heme macrocycle. Instead, the glutamate becomes a 6th axial ligand to the ferric heme iron producing a completely novel heme iron ligand set (Cys-Fe-Glu), with occupancy of the glutamate (instead of water) promoted by the binding of substrates to the P450.
EXPERIMENTAL PROCEDURES

Expression and purification of wild-type and mutant P450 BM3 proteins

Expression and purification of the mutant (A264E) and wild-type full-length flavocytochrome P450 BM3 and heme domains (amino acids 1-472) was performed essentially as described in previous publications (e.g. 30, 31). Expression plasmids pBM20 and pBM23 (wild-type P450 BM3 heme domain and intact flavocytochrome P450, respectively) and pHMG1 and pHMG2 (the respective heme domain and intact flavocytochrome P450 A264E clones) were expressed from *E. coli* strain TG1 grown in Terrific Broth plus 50 µg/ml ampicillin (typically 5 litres cells) for approximately 36 hours following inoculation from an overnight culture of the relevant transformant. Cells were collected by centrifugation, resuspended in Tris.HCl (50 mM, pH 7.2) plus 1 mM EDTA (buffer A) and broken using a French press (3 passes at 950 psi) followed by sonication of the resulting suspension on ice (Bandelin Sonopuls sonicator, 5 x 20 second pulses at 50 % full power, with adequate cooling time between pulses). Extract was exchanged by dialysis into ice-cold buffer A containing the protease inhibitors benzamidine hydrochloride and phenylmethylsulfonyl fluoride (PMSF, both inhibitors at 1 mM final concentration) prior to loading onto a DEAE column pre-equilibrated in the same buffer. Enzymes were eluted in a linear gradient of 0-500 mM KCl in buffer A. The most intensely red-coloured fractions were retained, concentrated by ultrafiltration (Centriprep 30 concentrators, Millipore) and dialysed extensively into 25 mM potassium phosphate (buffer B, pH 6.5, containing benzamidine and PMSF). Intact flavocytochrome P450 BM3 and its A264E mutant were loaded onto a mimetic yellow column (2 cm x 15 cm) and washed extensively in buffer B prior to elution with 25 mM 2’ and 3’ adenosine monophosphate (mixed isomers, Sigma) containing 500 mM potassium chloride. The wild-type heme domain and A264E mutant were loaded onto a hydroxyapatite column in buffer B, and eluted in a linear gradient of buffer B to 500 mM potassium phosphate (pH 6.5, plus protease inhibitors). The most intensely red-coloured fractions were pooled, concentrated and exchanged by dialysis into buffer A at 4 ºC, prior to loading on a Q-Sepharose column and eluting as described for the DEAE resin. All flavocytochrome and heme domains were pure at this stage (as judged by SDS PAGE analysis) and were concentrated by ultrafiltration to ≥500 µM, prior to dialysis into buffer A plus 50 % (v/v) glycerol and storage at
–80 °C. A264E mutant heme domain used in crystallographic trials were exchanged instead into 10 mM Tris.HCl (pH 7.2) and used directly for crystallogenesis.

**Site-directed mutagenesis of the CYP102A1 gene**

The A264E mutant forms of full length flavocytochrome P450 BM3 and its heme domain were constructed by overlapping mutagenic PCR. Three PCRs (A-C) were carried out in total, using the heme domain construct plasmid pBM20 (30) as template in PCRs A and B. The pBM20 construct contains the approx. 1.4 kb gene encoding the heme domain of P450 BM3 (amino acids 1-472 of the flavocytochrome) in the expression vector pUC118 (30). PCR A used the oligonucleotides MfeF (5’ CGCTTGATA_CAAATTGGTCTTTTGCGG 3’), incorporating an MfeI restriction site (underlined) 350 base pairs upstream of the mutation, and BmutR encompassing the mutagenic codon (5’ CCACTTGTTGTTCGTTCCCTCAATTAAG 3’, mutated nucleotide underlined). PCR B used the same template DNA and oligonucleotides, BMutF (5’ CTTAATTGA_GGGACACGAAACAACAAGTGG 3’) encompassing the mutagenic codon (mutated nucleotide underlined) and BamR, which incorporates a BamHI site (underlined) 335 bp downstream of the mutation (5’ CAGGTCGACTCTAGAGGATCCTATTAGCG 3’).

The final PCR reaction (PCR C) combined the products of PCRs A and B using the primers MfeF and BamR. The product of PCR C was A-tailed using Taq DNA polymerase and ligated into pGEM T according to the manufacturer’s instructions (Promega pGEM-T Easy Vector Systems). This yielded plasmid pGEM-A264E, which was completely sequenced by the dideoxy chain termination method at the Protein and Nucleic Acid Chemistry Laboratory (PNACL) facility at the University of Leicester. A MscI site upstream of the mutation was used in combination with MfeI to excise a 518 bp fragment containing the A264E mutated region. The relevant fragment was resolved on a 1 % agarose gel and purified using a QIAquick gel extraction kit (Qiagen). The MfeI/MscI restriction fragment was re-ligated into the backbones of pBM20 and pBM23 plasmids that had been digested with the same restriction enzymes and gel purified in the same way as the insert fragment. Correct insertion was verified by restriction enzyme digestion.
**SDS-PAGE analysis of A264E**

SDS-PAGE was performed using a Bio-Rad mini-protean II apparatus and either 10% or 6% polyacrylamide gels. SDS-PAGE was used to establish purity of the wild-type and mutant flavocytochromes P450 BM3 and heme domains, and also to resolve the A264E flavocytochrome P450 BM3 and heme domain prior to testing for covalent attachment between the heme and P450 protein. This was done using a heme-staining procedure to establish whether heme remained associated with the protein following denaturation in SDS (32).

The A264E heme domain was resolved on 10% SDS-PAGE gels in substrate-free and arachidonate-bound forms (~50 µM fatty acid), both in the absence of and following incubation with excess dithionite reductant. Intact A264E flavocytochrome BM3 was run on 6% SDS-PAGE gels in the same forms as for the heme domain, and also following incubation with excess NADPH (500 µM) in presence and absence of arachidonate, and following treatment with hydrogen peroxide (20 mM). Cytochrome c (horse heart, type IV, Sigma) was used as a positive control for covalent heme ligation in 10% gels, and *Shewanella frigidimarina* flavocytochrome c₃ was used in 6% gels (33). A total of 2 ng enzyme was run in each lane on the SDS PAGE gels along with protein molecular weight markers (NEB broad range protein marker). Gel preparation and electrophoresis were performed as described previously (34). To determine whether heme remained bound to the enzymes following denaturing gel electrophoresis (i.e. was covalently linked to the protein backbone). Gels were stained in the dark with 70 ml 0.25 M sodium acetate (pH 5.0), to which was added 30 ml of 6.3 mM 3,3',5,5'-tetramethylbenzidine (TMBZ) in methanol. Hydrogen peroxide (30 mM final concentration) was then added and the gel incubated until light blue bands appeared, indicating the presence of heme in certain proteins. The reaction was then stopped with the addition of isopropanol (approx. 10 % v/v). The gels were photographed and then re-stained with Coomassie blue, to ensure the presence of control and sample proteins at appropriate concentrations.

**Binding of substrates and ligands to wild-type and A264E P450 BM3**

Binding of fatty acids and heme-coordinating inhibitors to the wild-type and A264E heme domains was analysed by optical titrations using a Cary UV-50 Bio scanning spectrophotometer (Varian). The fatty acids arachidonate, palmitoleate, palmitate, myristate and laurate were used
in the titrations. The spectra for the substrate-free wild-type and A264E enzymes (typically 5-8 µM protein) were recorded at 30 °C in assay buffer (1 ml total volume of 20 mM MOPS, pH 7.4, plus 100 mM KCl), prior to additions of the fatty acids in aliquots of 0.1-0.5 µl (using a Hamilton syringe) up to a final volume of not more than 1 % of the total volume of the solution. Fatty acids were prepared as concentrated stocks (3-25 mM) in ethanol (arachidonate, palmitate and myristate) or DMSO (palmitoleate) or as a saturated aqueous stock in assay buffer (~ 900 mM) for laurate (31). Spectra were recorded after each addition of substrate, and a difference spectrum computed by subtraction of the starting (substrate-free) spectrum from those generated at each point in the titration. The maximal apparent absorption change induced at each point in the titration was determined by subtraction of the minimal absorption value at the trough in each difference spectrum from the maximal value at the peak (using data at the same wavelengths in each titration). The maximal changes in absorption determined in this way were plotted versus the relevant fatty acid concentration. A similar approach was taken to determine the apparent binding constant for the inhibitor 4-phenylimidazole. Data were fitted either to a rectangular hyperbola or, in cases where fatty acids bound very tightly to the P450, to a quadratic function that accounts for the quantity of enzyme consumed in the enzyme-substrate complex at each point in the titration (equation 1).

\[
A_{\text{obs}} = \left( \frac{A_{\text{max}}}{2Et} \right) \times \left( S + Et + K_d \right) - \left( \left( S + Et + K_d \right)^2 - \left( 4 \times S \times Et \right) \right)^{0.5}
\]

Equation 1

In equation 1, “A_{obs}” is the observed absorption change at substrate/ligand concentration “S”, “Et” is the total enzyme concentration and “K_d” is the dissociation constant for the enzyme-ligand/substrate complex. All fitting of data was done using Origin software (Microcal).

The “P450” form of wild-type and A264E mutant P450 BM3 were generated by addition of a few grains of fresh sodium dithionite to the proteins (typically 4-8 µM in assay buffer), followed by slow bubbling of the solution with carbon monoxide gas for approximately one minute. The nitric oxide adduct of the P450s was generated by release of ~ 5 small bubbles into a similarly buffered solution of ferric enzyme.
**Studies of effects of pH, ionic strength and temperature on optical properties of the A264E heme domain**

UV-visible spectra were recorded for the substrate-free form of A264E heme domain (4 µM) in 50 mM potassium phosphate in the pH range between 5.0-9.0, at 0.5 pH unit intervals. Spectral perturbations were observed and absorption data reflecting the maximal overall change between the low- and high-pH spectra ($\Delta A_{417}$ minus $\Delta A_{423}$ with reference to the spectrum collected at pH 7.0) were plotted against pH and fitted to a sigmoid to derive an apparent pK$_a$ value accompanying the spectral conversion. Further UV-visible spectra for the substrate-free form of A264E heme domain (4 µM) were collected in 20 mM MOPS (pH 7.4), and in the same buffer containing potassium chloride at concentrations between zero and 1 M. Finally, spectra were recorded for A264E heme domain (4 µM) in assay buffer at temperatures between 18-66 ºC in 2 ºC intervals. The sample was heated using a Peltier system on the Varian spectrophotometer, with temperature controlled via a circulating water bath attached to the Peltier. Two minutes equilibration time was allowed at each temperature point in the titration prior to collection of the spectrum.

**Redox Potentiometry**

All redox titrations were carried out in an anaerobic glove box (Belle technology, Portesham, England) under a nitrogen atmosphere, with oxygen levels maintained at $< 5$ ppm. Redox titrations were carried out for both wild type heme domain P450 BM3 and the A264E BM3 heme domain (typically 6-10 µM), both in the presence and absence of arachidonic acid (~ 70 µM, K$_d$ value is $< 5$ µM) by the method of Dutton, and essentially as described previously (12, 35, 36). Redox titration buffer (100 mM potassium phosphate, pH 7.0) was deoxygenated by bubbling extensively with O$_2$-free argon and degassed prior to transfer to the glove box. Oxygen was removed from P450 samples by passing concentrated stock solutions through a Bio-Rad Econo-pac 10DG gel filtration column in the glove box, which had been pre-equilibrated with redox titration buffer containing 5% (v/v) glycerol. Enzyme (typically 6-10 µM) was titrated with sodium dithionite as a reductant and potassium ferricyanide as an oxidant. Dithionite and ferricyanide were delivered in approximately 1-2 µl aliquots from concentrated stock solutions (typically 10-50 mM). Titrations were performed in both reductive and oxidative directions to
ensure lack of hysteresis. Absorption changes during the titrations were monitored via a fibre optic absorption probe (Varian) immersed in the P450 solution in the anaerobic environment, and connected to a Cary UV-50 Bio UV-visible spectrophotometer (Varian) outside the glove box. Potentials were measured using a Hanna pH 211 meter coupled to a Pt/Calomel electrode (ThermoRussell Ltd.) at 25 ± 2 °C. The electrode was calibrated using the Fe$^{3+}$/Fe$^{2+}$ EDTA couple as a standard (+108 mV). A factor of +244 mV was used to correct relative to the standard hydrogen electrode. Mediators were added to facilitate electrical communication between enzyme and electrode, prior to titration. Typically, 2 µM phenazine methosulfate, 5 µM 2-hydroxy-1,4-naphthoquinone, 0.5 µM methyl viologen, and 1 µM benzyl viologen were included to mediate in the range between +100 to –480 mV, as described previously (12, 35). The electrode was allowed to stabilize between each addition of reductant/oxidant prior to spectral acquisition and recording of the potential.

Data were analysed by plotting the absorbance at an appropriate wavelength, corresponding to the maximal absorbance change between oxidised and reduced forms, against the potential. A single electron Nernst function was then fitted to the data to describe the transition between ferric and ferrous heme iron, and the midpoint potential calculated from this data fit. For the substrate-bound titrations, arachidonate was added from a 33 mM stock in ethanol until no further change in spectral shift was observed. Data generated from the fitting procedures with wild-type P450 BM3 heme domain were in close agreement with previous studies (37).

**Steady-state kinetics**

The apparent rates of fatty acid-dependent NADPH oxidation catalysed by wild-type and A264E mutant flavocytochromes P450 BM3 were determined essentially as described previously (35). All measurements were carried out in a 1cm pathlength quartz cuvette in assay buffer at 30 ºC, with a final enzyme concentration of 0.1 µM. Reaction rates were determined over a range of fatty acid concentrations (using laurate, myristate, palmitate, palmitoleate and arachidonate, as for the optical titrations), typically up to approximately 100 µM fatty acid. The reaction was started with the addition of 200µM NADPH and activity monitored by ΔA$_{340}$ ($Δε_{340}$ = 6210 M$^{-1}$ cm$^{-1}$). Rates at individual substrate concentrations were measured in triplicate. Rate *versus*
substrate concentration data were fitted to a rectangular hyperbola to define the $K_M$ and $k_{cat}$ parameters.

Measurement of reductase domain-dependent reduction of cytochrome $c$ by wild-type and A264E mutant flavocytochromes P450 BM3 was performed essentially as described above for fatty acid turnover. However, enzyme concentration was 7 nM, and rates were determined from the accumulation of reduced products at appropriate wavelengths: for ferricyanide at 420 nm ($\Delta\varepsilon_{420} = 1010 \text{ M}^{-1} \text{ cm}^{-1}$) and for cytochrome $c$ at 550 nm ($\Delta\varepsilon_{550} = 22640 \text{ M}^{-1} \text{ cm}^{-1}$) (38).

**Determination of oxygenated fatty acid products**

Turnover experiments were carried out by incubating 0.4 $\mu$M wild-type or A264E flavocytochrome P450 BM3 with 200 $\mu$M myristic acid, palmitic acid or palmitoleic acid, and 600 $\mu$M NADPH in a final volume of 5 ml. The reaction was allowed to proceed for 4 hours at 30 °C with stirring, before halting the reaction by acidification to pH 2.0 with hydrochloric acid. Fatty acids were extracted from the aqueous environment into one volume of dichloromethane. Remaining aqueous material was removed by the addition of excess solid magnesium sulphate to the mixture. Following filtration, to remove magnesium sulfate, the dichloromethane was evaporated under a stream of nitrogen gas and lipids resuspended in a small volume of methanol. Thereafter, 20 $\mu$l samples were analysed by electrospray mass spectrometry (70 eV ionisation) using a Micromass Quattro triple quadrupole mass spectrometer. Samples from the aqueous layer were also run to ensure complete extraction and negative controls (in which no NADPH was added to the enzyme/fatty acid mixtures) were also examined to ensure that any products resulted from NADPH-dependent enzyme activity.

**Spectroscopic analysis**

**EPR spectroscopy**: Perpendicular mode X-band Electron paramagnetic resonance (EPR) spectra were recorded on an EPR spectrometer comprising an ER-200D electromagnet and microwave bridge interfaced to an EMX control system (Bruker Spectrospin) and fitted with a liquid helium flow-cryostat (ESR-9, Oxford instruments) and a dual-mode X-band cavity (Bruker type
Novel heme ligation in a cytochrome P450

ER4116DM). Spectra were recorded at 10 K with 2 mW microwave power and a modulation amplitude of 1 mT. Protein samples were in 20 mM MOPS (pH 7.4) plus 100 mM KCl.

EPR spectra were recorded for wild-type (~ 300 µM) and A264E (~ 400 µM) heme domains of P450 BM3 in absence of added substrate, in the presence of the substrate arachidonic acid (500 µM) and in the presence of the azole inhibitor 4-phenyl imidazole (500 µM). Saturation with substrate/inhibitor was verified by acquisition of UV-visible spectra (using a 0.1 cm pathlength cuvette).

Circular Dichroism: CD spectra were recorded in the far UV (190-260 nm) and near UV-visible (260-600 nm) ranges using a Jasco J715 spectropolarimeter instrument at ambient temperature. Spectra were recorded for both substrate-free and for substrate- (arachidonate) bound forms of the A264E heme domain (0.25 µM P450 in the far UV region; 20 µM P450 in the near UV-visible region) in assay buffer. Spectra were recorded in quartz cells of 0.1 cm and 1.0 cm pathlength for far UV and near UV-visible regions, respectively, at scan rates of 10 nm/min for the far UV region and 20 nm/min for the near UV-visible region. Arachidonic acid (100 µM) was delivered in ethanol (final concentration <0.5 % v/v). Control spectra were recorded for P450 BM3 A264E in the presence of ethanol alone to ensure no effect induced by the solvent.

Magnetic Circular Dichroism spectroscopy: Magnetic circular dichroism (MCD) spectra were recorded for wild-type and A264E P450 BM3 heme domains on circular dichrographs, JASCO models J-810 and J-730 for the UV-visible and near-infrared regions, respectively, using a 0.1 cm pathlength quartz cuvette and the same sample concentrations used for the EPR studies. An Oxford instruments superconducting solenoid with a 25 mm ambient bore was used to generate a magnetic field of 6 Tesla for the room temperature MCD measurements. At room temperature, MCD intensities are linearly dependent on magnetic field and are plotted normalized to magnetic fields as $\Delta \varepsilon / H$ (M$^{-1}$ cm$^{-1}$ T$^{-1}$). In order to record MCD through the 1400-2000 nm region, interference from absorptions caused by vibrational overtones was minimized by preparing samples in deuterium oxide solutions (39). Samples were prepared in 20 mM HEPES made up in deuterium oxide [pH 7.5]).
**Resonance Raman spectroscopy:** Resonance Raman spectra for P450 BM3 A264E heme domain were obtained in 0.1 M potassium phosphate (pH 7.0) using 15 mW, 406.7 nm radiation at the sample, from a Coherent Innova 300 Krypton ion laser. Spectra were acquired using a Renishaw micro-Raman system 1000 spectrometer. The sample (approximately 50 µM) was held in a capillary under the microscope, with 8 x 30 seconds exposure for data collection. Spectra were recorded in the absence of substrate, and following addition of arachidonic acid (100 µM) to the sample.

**RESULTS**

**Molecular biology, expression and heme-binding properties**

The A264E mutations were introduced into wild-type constructs of full length flavocytochrome P450 BM3 and its heme domain by overlapping PCR mutagenesis as described in the *Experimental* section. In the final constructs the presence of the correct mutation (and the absence of exogenous mutations) was verified by complete sequencing of the genes by the dideoxy method. Levels of expression of the A264E flavocytochrome P450 BM3 and heme domains were not significantly perturbed by the I helix mutation A264E, with typically 20-30 mg of protein produced per litre of *E. coli* transformants. Mutant flavocytochrome P450 and heme domains were purified as described previously (21, 31), and preliminary characterization by UV-visible spectroscopy indicated that the oxidised form of the pure enzymes incorporated heme fully and had Soret/A<sub>280</sub> ratios very similar to the wild-type forms. To establish whether the enzymes purified from *E. coli* had covalently attached heme, wild-type and A264E protein samples (heme domain and full length flavocytochrome) were resolved by SDS-PAGE and stained using TMBZ according the methods described by Thomas *et al.* (32). In contrast to the results of Rettie and of Ortiz de Montellano with various CYP4 isoforms (23-26), there was no discernible staining of BM3 heme with TMBZ that would indicate the presence of covalently bound heme. In view of previous results indicating turnover-dependent formation of the covalent linkage in CYP4 isoforms, the A264E heme domain and full length flavocytochrome were pre-incubated both with and without excess arachidonic acid, and plus/minus excess reductant (dithionite or NADPH). Additional samples (plus/minus arachidonate) were exposed to hydrogen...
peroxide (which can drive P450 catalysis via the “peroxide shunt” pathway). All samples were incubated at ambient temperature or 30 °C for periods between 5-30 minutes for reactions to occur. Samples so treated were again resolved by SDS-PAGE and stained for heme. However, again there was no evidence of any detectable amount of covalently bound heme. Controls with wild-type enzymes produced similar results. By contrast, strong blue bands were observed for the two control hemoproteins used: horse heart cytochrome \textit{c} and \textit{Shewanella frigidimarina} flavocytochrome \textit{c}_3. Both these enzymes contain covalently attached \textit{c}-type heme groups (33). Subsequent staining of these gels with Coomassie blue confirmed the presence of large amounts of the relevant P450 proteins, providing further proof that the \textit{b}-type heme did not remain bound to the proteins in either wild-type or A264E forms.

\textbf{UV-visible spectroscopy}

Despite the apparent lack of covalent heme ligation, close examination of the electronic spectrum of the oxidized forms of the A264E enzymes indicated that the heme signals were slightly shifted with respect to the wild-type forms. The Soret band maximum for both oxidized, substrate-free A264E flavocytochrome and heme domains was shifted to longer wavelength by approximately 1-2 nm with respect to the wild-type forms (from ~ 418 nm to 420.5 nm), and there were similar small perturbations in the \textit{α}/\textit{β} band region (from ~ 568 nm to 571 nm for the \textit{α} band; from 534 nm to 538 nm for the \textit{β} band). In the dithionite-reduced form, the spectral properties of the ferrous forms of the substrate-free wild-type and A264E mutants were virtually indistinguishable, with the Soret band shifted to 411 nm and the \textit{α}/\textit{β} bands apparently fused with a maximum at 546 nm (\textbf{Figure 2A}). The spectral properties of the ferrous-carbon monoxy complexes were also determined, showing a Soret shift to ~ 449 nm for the A264E heme domain cf ~ 448 nm for the wild-type enzyme (30, 40). To analyse further the spectral properties of the A264E mutant, a nitrosyl complex was generated by bubbling the A264E heme domain with NO gas (5 small bubbles). A Soret band shift to 435 nm was observed (\textit{cf} 434 nm for wild-type BM3 heme domain). As with wild-type enzyme, there was a marked increase in intensity of the alpha and beta bands, with their maxima shifted to 575 nm and 543.5 nm, respectively. The binding of a tight-binding azole inhibitor (4-phenylimidazole) was also performed. In this case, the spectral features of the complex were virtually indistinguishable from those of wild-type P450 BM3, with
the Soret band shifted to 425.5 nm, and with changes in alpha/beta band intensity and shifts to 575/544 nm (Figure 2B).

FIGURE 2 HERE

To examine spectral effects on the binding of fatty acid substrates, optical binding titrations were done for the A264E heme domain using a number of fatty acids (lauric acid, myristic acid, palmitic acid, palmitoleic acid and arachidonic acid) known to bind tightly to the wild-type enzyme. Surprisingly, spectral changes observed on addition of fatty acids to A264E were distinct from those observed previously for wild-type P450 BM3 and various mutant forms (e.g. 31). Neither lauric acid or myristic acid (C12 and C14 saturated fatty acids, respectively) induce any significant change in the spectral properties of A264E mutant, even at concentrations near their solubility limits (950 µM and 250 µM, respectively). By contrast, the longer chain fatty acids palmitic acid (C16 saturated), palmitoleic acid (C16 mono-unsaturated) and arachidonic acid (C20, polyunsaturated) all induced spectral changes. However, rather than inducing a type I optical change with shift of the Soret maximum to shorter wavelength, a Soret shift to a final wavelength maximum of approximately 426 nm was observed in all cases. The final extent of the spectral shift induced is dependent on the particular fatty acid used, with arachidonic acid being the most effective amongst those tested. This aspect of the mutant’s behaviour is discussed in more detail in the Discussion section. Thus, rather than undergoing a substrate-dependent optical transition typical of increased high-spin heme iron content, the A264E mutant shows instead a type II transition usually observed on ligation of inhibitors to the heme iron (e.g. imidazoles, see Figure 2B). This type of optical transition likely indicates reinforcement of the low-spin form of the cytochrome. Data for the optical titration with arachidonic acid are shown in Figure 3.

FIGURE 3 HERE

Notwithstanding the unusual spectral changes observed, the apparent binding constants (Kd values) were determinable from plots of induced spectral changes versus fatty acid concentration, as detailed in the Experimental section. The Kd values determined for the binding of the fatty acids arachidonic acid, palmitic acid and palmitoleic acid to the A264E heme domain
are lower than those determined from optical titrations with the same fatty acids and the wild-type heme domain (Table 1). While myristic acid and lauric acid failed to produce spectral changes of sufficient magnitude to facilitate any accurate determination of their apparent $K_d$ values for A264E heme domain, the same fatty acids do induce spectral conversion of the wild-type enzyme. However, it should be noted that the extent of laurate- and myristate-induced spin-state conversion in the wild-type P450 BM3 heme domain is lower than that produced with the other fatty acids tested here. It should also be noted that the apparent $K_d$ values determined for palmitate, palmitoleate and arachidonate with A264E heme domain are all considerably tighter than those for the wild-type heme domain (Table 1).

**TABLE 1 HERE**

An observation from studies of the pH-dependence of the UV-visible electronic spectrum of the A264E mutants was that the absorption maximum of the Soret band was very sensitive to pH changes in the range between 5-9.5 (in which there was not any significant destruction of the heme). Spectra for the A264E heme domain were recorded in potassium phosphate at several pH values across this range. At pH 7.5, the Soret maximum was located at 420.5 nm, as seen in the buffer used for fatty acid-binding titrations and kinetic studies (e.g. see Figure 2). As the pH was lowered, there was a consistent shift in the absorption maximum of the Soret band towards shorter wavelength, reaching 418 nm at pH 5. At higher pH values, the Soret maximum moved to longer wavelengths, positioning at 423 nm at pH 9 and 9.5. A plot of the peak position of the Soret band versus the solution pH described a sigmoid, suggesting the importance of a single ionisable group and possibly reflecting the $pK_a$ value for a protonatable amino acid sidechain. The apparent $pK_a$ value determined for the optical transition observed was 8.0 ± 0.2. To examine further the sensitivity of the spectrum of the A264E heme domain to solution conditions, spectra were recorded for the wild-type and A264E enzymes in standard assay buffer (20 mM MOPS [7.4] + 100 mM KCl) and in the same MOPS buffer with KCl in the range between 0-1 M in 100 mM intervals. Increasing ionic strength produced negligible effects on the spectrum of wild-type P450 BM3 heme domain, but induced spectral changes in the A264E heme domain similar to those observed at high pH, i.e. a shift of the Soret band to longer wavelength. In 20 mM MOPS (7.4), the A264E Soret band was centred at ~419 nm, moving to 420.5 nm in the typical P450
Novel heme ligation in a cytochrome P450 BM3 assay buffer (i.e. plus 100 mM KCl) and to progressively longer wavelengths at higher [KCl]. By 500 mM KCl, the Soret band had shifted as far as 422 nm, and at 1 M KCl it was further red shifted to ~423.5 nm. Thus, both high pH and elevated ionic strength produce type II optical shifts in A264E heme domain (and intact flavocytochrome) that are comparable with those induced by addition of long chain fatty acids to the mutant, and which are indicative of a novel ligation state of the heme iron.

Steady-state kinetic parameters
The kinetic properties of wild-type and A264E flavocytochromes P450 BM3 were determined with respect to their capacity to catalyse the fatty acid-dependent oxidation of NADPH and the reduction of an exogenous electron acceptor (cytochrome c). Preliminary studies of the pH-dependence of the reaction of both the wild-type and A264E enzymes with arachidonic acid indicated that the rate was maximal at pH 7.5 ± 0.2, thus kinetics were studied in the standard assay buffer we have used in several previous studies of P450 BM3 (20 mM MOPS [7.4] + 100 mM KCl). As expected, the A264E mutant catalysed rapid NADPH-dependent reduction of cytochrome c (mediated via the FMN domain of the enzyme), indicating that the diflavin reductase domain of the enzyme is catalytically unimpaired by the mutation in the heme domain. However, rates of fatty acid-dependent NADPH oxidation were considerably slower than those for the wild-type P450 BM3 (Table 1). In the absence of fatty acids, both wild-type and A264E flavocytochrome P450 BM3 oxidise NADPH at a slow rate (~ 5 min⁻¹). In the presence of either lauric acid or myristic acid, there was considerable stimulation of NADPH oxidase activity, despite the fact that addition of these fatty acids did not induce any considerable changes in the optical spectrum of the A264E mutant. Enzyme activity in the presence of palmitic acid, palmitoleic acid or arachidonic acid was even higher, albeit rather less than that observed with wild-type P450 BM3. (Table 1). Thus, it appears that the flavocytochrome P450 BM3 A264E mutant retains considerable levels of activity with long chain fatty acids, despite the fact that these fatty acids induce either negligible change towards high spin, or inhibitor-like optical change to the heme spectrum.
Fatty acid oxygenation

In view of the apparently conflicting data indicating high levels of fatty acid-dependent NADPH oxidation despite type II optical shifts induced by the same fatty acids, we undertook studies to establish whether NADPH oxidation was linked to oxygenation of myristic acid, palmitic acid and palmitoleic acid. Turnover studies were performed as described in the Experimental section, and products were examined by mass spectrometry. Products were evident from turnover of each of these substrates. In the case of myristic acid, there was similar levels of production of monooxygenated product for both wild-type and A264E flavocytochrome P450 BM3. With palmitic acid, mass spectrometry showed the presence of both mono- and di-oxygenated products for both wild-type and A264E enzymes, consistent with previous data and indicative that a primary hydroxylated product can act as a substrate for a second round of oxidation (41, 42). With the mono-unsaturated fatty acid palmitoleic acid (cis-9-hexadecenoic acid) as the substrate, both mono- and di-oxygenated products were observed for both wild-type and A264E enzymes, again with rather lower amounts of products with the mutant enzyme. In previous studies, Fulco and co-workers demonstrated that both hydroxylation (close to the \( \omega \)-terminus) and epoxidation (across the C\( _9 \)-C\( _{10} \) double bond) of palmitoleic acid are catalysed by wild-type P450 BM3 (43, 44). Under the same experimental conditions, the amounts of products generated from palmitic acid and palmitoleic acid by the A264E mutant were \( \sim 30-40\% \) lower than those produced by wild-type P450 BM3, consistent with the differences in steady-state kinetics shown in Table 1. In parallel studies of peroxide production during fatty acid turnover, there was no significant difference between the wild-type and A264E enzymes, suggesting that both enzymes couple NADPH oxidation to fatty acid oxygenation tightly, but that the A264E mutant is a much slower hydroxylase than the wild-type P450 BM3 (45). Thus, despite the unusual spectral conversions produced on binding long chain fatty acids, the A264E flavocytochrome retains the capacity to oxygenate fatty acids.

Spectroscopic analysis

Optical binding studies for the A264E heme domain indicate that the resting, oxidized form of the enzyme has a perturbed UV-visible spectrum from that seen in the wild-type enzyme, and that substrate addition, basic \( \text{pH} \) and high ionic strength induce optical changes indicative of a change in heme iron coordination state. While no detectable covalent ligation of the heme
Novel heme ligation in a cytochrome P450 21 macrocycle was detectable with the A264E flavocytochrome P450 BM3, it appeared likely that the glutamate may instead interact with the heme macrocycle or, more likely, coordinate to the heme iron itself to produce the spectral perturbation observed. For this reason, we undertook spectroscopic analysis to examine structural features of the mutant protein and its bound heme cofactor.

**Circular Dichroism:** Far UV CD spectra (190-260 nm) for the substrate-free A264E heme domain indicate no significant change in secondary structure compared with the wild-type heme domain. The predominantly alpha helical A264E heme domain has a far UV CD spectrum virtually identical to that for the wild-type enzyme, with minima at 223 nm and 209 nm, and an abcissa at 202 nm. The far UV CD spectrum for A264E (as with the wild-type) is not significantly altered by addition of arachidonic acid, showing that secondary structural content is not significantly altered on binding the substrate. However, in the near UV-visible CD region (260-600 nm), the spectrum of the A264E heme domain in its resting, oxidized form is rather different from that for wild-type heme domain. This portion of the spectrum is dominated by a sharp Soret feature with negative ellipticity. For the substrate-free wild-type heme domain this is centred at ~409 nm, and shifts to ~404 nm on arachidonic acid binding (**Figure 4**). Thus, the direction of movement of the Soret band (to shorter wavelength) mimics the optical transition seen in the electronic absorption spectrum. However, the near UV-visible CD spectrum of the substrate-free A264E heme domain has its Soret band at ~422 nm. On addition of arachidonic acid, this feature sharpens and shifts to 426 nm, the same wavelength as seen for the Soret maximum in the optical spectrum of arachidonate-bound A264E heme domain (**Figure 4**). Thus, near UV-visible CD spectroscopy shows much more marked differences in the spectra of wild-type and A264E heme enzymes than does optical absorption spectroscopy. A large spectral difference is seen between the near UV-visible CD spectra substrate-free forms of wild-type and A264E heme domains, whereas (under identical conditions) optical absorption shows a shift of only ~1.5 nm in the Soret band.

**FIGURE 4 HERE**
Resonance Raman: Resonance Raman spectra were recorded for the substrate-free and arachidonic acid-bound forms of the A264E heme domain. For wild-type P450 BM3, considerable spectral perturbations are observed on addition of fatty acids, including shifts in position and intensity of the spin-state marker bands $\nu_2$ and $\nu_{10}$, reflecting the change in heme iron spin-state equilibrium towards the high-spin form (30). By contrast, there are no significant changes in any of the oxidation (e.g. intensity of $\nu_4$ remains constant at 1371 cm$^{-1}$) or spin-state (e.g. intensity of $\nu_3$ remains constant at 1501 cm$^{-1}$) marker bands for the A264E heme domain following addition of arachidonic acid. Resonance Raman confirms that the substrate-free enzyme is essentially completely low-spin, and that addition of arachidonate reinforces the low-spin state. The only significant differences following fatty acid addition to the A264E heme domain are small increases in intensity of the $\nu_{C=C}$ band at 1621 cm$^{-1}$, relating to the heme vinyl groups, and of the $\nu_{11}$ band at 1560 cm$^{-1}$. The $\nu_{11}$ band is affected by the degree of conjugation of the heme vinyl groups and their planarity with the heme macrocycle, and these small perturbations should reflect alterations in the positioning of the vinyl groups relative to the heme plane following fatty acid binding. However, resonance Raman does not provide specific vibrational information on the proposed switch of distal ligands to the heme iron that occurs on arachidonic acid binding to the A264E heme domain. For such information, we turned instead to EPR and MCD spectroscopy.

EPR:
EPR spectroscopy of the arachidonate-bound and substrate-free forms of the wild-type and A264E heme domains were recorded, and are shown in Figure 5A. The EPR spectrum for wild-type P450 BM3 is as previously reported (46) and typical for low-spin ferric P450s which all give rise to spectra with $g_z$ in the range 2.40-2.45 (47-49). On binding of substrate, the ferric spin equilibrium is perturbed and the iron becomes a mixture of low- and high-spin. The latter appears in the EPR with features at $g = 8.18$, 3.44, 1.66 which originate in the lowest ($m_s = \pm \frac{1}{2}$) Kramers doublet of the $S = 5/2$ ferric ion. In the “low-field limit” where the axial zero-field-splitting parameter is greater than the Zeeman splittings (D $>>$ gβH) these values correspond to a rhombicity of E/D $= 0.11$. In ferric hemoproteins, such substantial rhombicities are found only with thiolate ligation. The EPR spectra of the substrate-free and arachidonate-bound A264E mutant of P450 BM3 both suggest the presence of several low-spin ferric species,
all with g-values which indicate that thiolate coordination is maintained. A264E appears to contain two distinct forms with \( g_z \sim 2.56 \) and 2.43 respectively. Each feature shows structure indicative of further minor heterogeneities. There is no example of cysteinate proximal to a neutral oxygen ligand giving a \( g_z \)-value greater than 2.45. The \( g_z \sim 2.56 \) must therefore indicate ligation different from that of the wild-type and is strong evidence for coordination of the distal glutamate. In support of this, the formate derivatives of P450 cam and chloroperoxidase give \( g_z \) at 2.55 and 2.59 respectively (50, 51). The feature at \( g_z \sim 2.43 \) suggests that a proportion of the sample retains a distal water ligand although it appears that the distal mutation causes some heterogeneity in this sub-population. In contrast to the wild-type P450 BM3 enzyme, when substrate is bound to the A264E mutant, there is no significant switch to a high-spin form. Instead, changes in the EPR spectrum for arachidonate-bound A264E indicate differences in the distribution of the low-spin species, with a diminution of the contribution from a Cys-aqua ligated form and a simultaneous increase in the proportion of Cys-Glu coordinated species, indicating that substrate-binding promotes the ligation of Glu 264 to the heme iron.

**FIGURE 5 HERE**

A comparison of the EPR spectra for 4-phenylimidazole-bound forms of wild-type and A264E mutant heme domains is shown in Figure 5B. There is some heterogeneity in the wild-type complex, with the major triplet of g-values at 2.57, 2.26 and 1.86/1.85. The \( g_z \) signal is broadened, suggesting that there may be a split population of two conformers with a slightly different \( g_z \) component, and paired with the two subtly different \( g_x \) components. Minor signals at 2.44 and 1.92 may reflect a small proportion of non-ligated enzyme. In theazole-bound A264E complex, there appears to be one predominant species with g-values at 2.58, 2.26 and 1.86. The homogeneity of this spectrum is in part due to the apparently complete ligation of the azole to the heme iron in this sample (removing residual aqua-ligated components seen in the wild-type spectrum), consistent with the tighter \( K_d \) value determined for binding of 4-phenylimidazole to A264E (c.f. wild-type) from spectral titrations (see Table 1). However, it appears to be the case that a single conformational form of azole-bound heme is present in A264E, whereas there may be two distinct species in the wild-type P450.
The MCD spectra for wild-type P450 BM3 heme domain are consistent with those we have reported earlier (46). The room temperature near UV-visible MCD spectra of substrate-free wild-type and A264E P450 BM3, and the arachidonate-bound A264E enzyme (Figure 6A) each show a pattern of bands typical for low-spin ferric hemes with a thiolate ligand. The unusually low MCD intensity in both the Soret band (400-420 nm) and the $\alpha/\beta$ region (500-600 nm) is also characteristic of such species (52-61). A small additional negative feature, at ~655 nm, is part of a derivative-shaped charge-transfer band and arises from the presence of a low level ($\leq$ 15%) of the high-spin form (see 62). The spectrum of substrate-bound wild-type P450 BM3 is very different and shows that the low-spin heme is now the minority species (~25%). The CT band near 655 nm has increased in intensity and other high-spin bands are evident at 360-405 nm, and as a shoulder at ~555 nm. Low-spin ferric hemes also give rise to a porphyrin-to-ferric charge-transfer transition at longer wavelengths. This appears as a positive signed band in the MCD and has been located in the room temperature NIR MCD for wild-type P450 BM3 minus substrate, and for the A264E mutant in both the absence and presence of substrate at ~1080 nm (Figure 6B) as was previously reported for WT BM3-P450 at low-temperature (46). Close inspection of these three spectra reveals qualitative and quantitative differences. The peak position for the transition shifts from ~ 1075 nm for the wild-type substrate-free enzyme, through to ~1080 nm for the substrate-free A264E mutant, and to ~1085 nm for the arachidonate-bound A264E mutant. Differences in both the breadth and intensity of the CT band are discernible (Figure 6B). The exact energy of this CT transition is generally diagnostic of the two heme axial ligands (63, 64), but the influence of the second ligand is somewhat reduced in the presence of thiolate, as illustrated here by these three examples. Changing water for carboxylate at the distal side of the heme results in only very minor band shifts. For imidazole-bound P450, native CooA and the hemes in subunit I of SoxAX, all of which have a nitrogenous ligand distal to cysteinate, the CT transitions are at 1180 nm (46), 1120 nm (63) and 1150 nm (65) respectively.

**FIGURE 6 HERE**

Addition of arachidonic acid substrate to wild-type P450 BM3 results in a marked change in the NIR MCD spectrum. Consistent with the switch to predominantly high-spin heme that was
observed at UV-visible wavelengths, the low-spin CT band near 1100 nm is significantly diminished. The derivative-shaped MCD band centred at ~900 nm is the CT band characteristic of high-spin ferric heme and is extremely similar to that reported for substrate-bound cytochrome P450 cam (66).

**Potentiometric analysis**

Previous studies have shown that fatty acid binding to wild-type P450 BM3 is accompanied by loss of the aqua ligand to the heme iron and a shift in the heme iron spin-state equilibrium towards the high-spin form (21, 30). In P450 BM3 (as in P450 cam), this is accompanied by a change in the heme iron reduction potential of ~130-140 mV (from −427 mV to −289 mV for P450 BM3) (12, 37). In view of the markedly different effect of fatty acid binding to the A264E variant, we undertook potentiometric studies of the substrate-free and arachidonic acid-bound forms of this mutant. For both forms of the enzyme, complete dithionite-dependent reduction of the heme iron proved facile under anaerobic conditions, and even under aerobic conditions the A264E heme iron was almost stoichiometrically reduced by addition of excess dithionite. By contrast, it is difficult to reduce the substrate-free wild-type heme domain completely using dithionite, and impossible under aerobic conditions due to its negative potential and rapid reoxidation of the ferrous form. On addition of near-saturating arachidonate, the A264E Soret band is located at approximately 426 nm, and also shifts to a final position of ~410 nm on complete reduction of the heme iron during the redox titration (Figure 7A). In the substrate-free form, the Soret band of the oxidized A264E heme domain is located at 419.5 nm, and shifts progressively to a final position of approximately 410 nm on complete reduction of the heme iron (Figure 7A). Absorption *versus* potential data were plotted at 410 nm, and were fitted to a single electron Nernst equation to define the midpoint potential for the A264E heme iron in the presence and absence of arachidonate. These values were −316 ± 3 mV in the substrate-free form and −314 ± 4 mV in the arachidonate-bound form. Thus, substrate-binding (which does not increase the high-spin content in the A264E enzyme) does not induce any significant alteration of the apparent reduction potential in the A264E heme domain under these conditions.

**FIGURE 7 HERE**
DISCUSSION
The capacity of the eukaryotic family 4 cytochromes P450 to link their heme macrocycle covalently to the protein backbone has been one of the most significant discoveries in P450 research in recent years (e.g. 23, 24). From a biotechnological perspective, the ability to covalently link the porphyrin to the P450 protein matrix is attractive for at least two reasons. Firstly, the presence of the glutamate and the glutamate ligation process was shown to enhance catalytic activity in rabbit CYP4B1 (26), and enhancement of catalytic rate is clearly a desirable feature to endow on an enzyme. Secondly (and the major reason), the capacity of P450s to undergo conversion to the inactive “P420” form (in which native cysteinate heme ligation is lost) is well recognized, and heme can even be dissociated completely from the P450 enzyme under moderately denaturing conditions (e.g. 67). Thus, the ability to covalently tether heme should promote longevity of P450 activity, particularly since the P450/P420 conversion has been shown to be reversible in selected P450s, including P450 BM3 (e.g. 68). From a perspective of exploitation of P450 enzymes, the bacterial enzymes P450 cam and P450 BM3 are the most intensively studied. Rational mutagenesis of P450 cam has produced variants of the camphor hydroxylase that are able to oxygenate molecules such as butane and propane (69). Rational mutagenesis of the fatty acid hydroxylase P450 BM3 has produced variants that catalyse oxygenation of fatty acids at different positions from the wild-type form, and in which substrate selectivity has been converted towards short chain fatty acids and polycyclic aromatic hydrocarbons such as phenanthrene (21, 70). Forced evolution of P450 BM3 has also produced an efficient alkane hydroxylase enzyme (22). It is clear that P450 BM3 has great biotechnological potential for production of functionalised hydrocarbons. In view of this potential and the close relationship between P450 BM3 and the eukaryotic family 4 fatty acid hydroxylases, we mutagenised P450 BM3 to introduce the glutamate conserved in the eukaryotic family 4 P450s at the same I helix position in P450 BM3 (i.e. Ala 264) in order to examine whether a similar covalent heme linkage could be induced in P450 BM3. It is clear that the heme linkage does not occur in this point mutant. However, unusual spectral perturbations were evident in the A264E variant, and these suggested that the glutamate residue might instead interact directly with the heme iron. This theory was validated by a combination of spectroscopic methods, and ultimately by obtaining the atomic structure of the A264E heme domain in substrate-free and palmitoleate-bound forms (see accompanying paper by Joyce et al., 71).
The capacity of long chain fatty acids to induce a type II spectral transition of the P450 heme in the A264E heme domain and flavocytochrome was the first indication that the introduced glutamate might replace water as the distal ligand to the heme iron in the long chain fatty acid-bound forms of the enzyme. In turn, this suggested that the reason for the perturbed spectrum in the substrate-free form of the A264E enzyme (Soret band at 420.5 nm c.f. 419 nm in wild-type P450 BM3) was that a proportion of the heme iron was glutamate ligated in the resting enzyme, and that the equilibrium was forced towards the glutamate-ligated form in the presence of long chain fatty acids (the preferred substrates for P450 BM3). Increasing ionic strength and basic pH have a similar effect on the optical spectra, and in the latter case an apparent pKₐ of 8.0 ± 0.2 may be assigned to the protonation of Glu264 in its hydrophobic location in the mutant active site. The fact that the shorter chain fatty acids (myristic acid, lauric acid) have almost negligible effect on the ligation state is consistent with their weaker binding to wild-type P450 BM3, and is also explicable in terms of their positioning in the active site of the enzyme. Li and Poulos determined the atomic structure of palmitoleate-bound, oxidised wild-type P450 BM3, and highlighted the interaction of the carboxylate group with the Ty51/Arg47 motif near the mouth of the substrate entry channel (72). The α-terminus of the longer chain fatty acids will extend further towards the heme iron in the oxidized form of P450 BM3 and the A264E variant, and this is predicted to afford their interaction (directly or indirectly through the influence of residue Phe87) with the glutamate, and to induce its repositioning to interact with the heme iron. The shorter chain fatty acids, their carboxylate still tethered at the active site mouth, are predicted not to extend far enough towards the heme to enable interaction with the glutamate. Again, these predictions are supported by structural data (see accompanying paper by Joyce et al., 71).

Spectroscopic studies were undertaken to provide further proof that a novel form of heme iron ligation occurred in the P450 BM3 A264E variant. Resonance Raman provides data consistent with those obtained from optical titrations, confirming that the low-spin form is reinforced on substrate binding. However, it cannot provide convincing data regarding the proposed ligand switch in A264E. EPR and MCD spectroscopies can provide such data. EPR studies of wild-type P450 BM3 heme domain are consistent with previous work on the enzyme, and show that the binding of fatty acid induces a large change in spin-state equilibrium towards the high-spin form.
However, the spectra for the A264E mutant are considerably different from those for the wild-type heme domain. In the absence of substrate the EPR spectrum of A264E is typical for a low-spin heme, but shows substantial heterogeneity in the signal that we consider result from an ensemble of forms present. The observation of g-values identical to those of the substrate-free wild-type heme domain indicates that a significant proportion of the mutant persists in a “native-like” water-bound form, although small shoulders on these features suggest some minor heterogeneity (Fig. 5A). The other three $g_z$ components clustered around $g \approx 2.56$ are assigned to glutamate-ligated forms of the protein. This three-fold heterogeneity may result from different orientations of the glutamate distal ligand, and possibly from interactions between the two different oxygen atoms of the glutamate carboxylate and the heme iron.

Atomic structural data shows that, in the aqua-ligated form of A264E, the glutamate residue interacts with the sidechain of phenylalanine 87 (see accompanying paper by Joyce et al., 71). Phe87 is known to be a conformationally flexible amino acid, which is important in controlling substrate selectivity and for interaction with the $\omega$-terminal carbon of fatty acid substrates of P450 BM3, preventing hydroxylation at this position (72, 73). Potentially, interactions between Phe87 and Glu264 in its ligated form provide further explanations for the heterogeneity in the substrate-free EPR spectrum. In the arachidonate-bound form of A264E the heterogeneity is reduced, due partly to the loss of much of the aqua-ligated form as the fatty acid “drives” on the glutamate ligand. It is likely that this occurs due to the substrate displacing unligated glutamate from its other favoured conformation, in which it interacts with the phenyl group of Phe87. However, the apparent degree of heterogeneity in the EPR spectrum is further reduced due to alterations in the proportions of the different glutamate-ligated forms of A264E. Potentially, this also reflects the re-positioning of Phe87 following binding of substrate, and the position(s) adopted by the fatty acid itself. The EPR data for the glutamate-ligated form(s) of P450 BM3 are consistent with previous studies of P450 cam in complex with oxygen donor ligands (e.g. formate, acetate and propionate), and there is particularly strong similarity to the formate-bound P450 cam, which displays g-values at 2.55, 2.25 and 1.88 (50, 53).

A limited number of heme iron ligand sets are observed in natural cytochromes. These include His-Met (e.g. in cytochrome $cd1$ from Pseudomonas aeruginosa), Met-Met (bacterioferritin from
the same bacterium) and His-His (e.g. eukaryotic cytochrome b₅) (74-76). Cysteinate-ligated heme enzymes include the Cys-aqua ligated cytochromes P450 and the nitric oxide synthases (e.g. 46, 77) and the Cys-His ligated heme is the SoxAX protein from *Rhodovulum sulfidophilum*, with a role in thiosulfate oxidation (65). However, the Cys-Glu ligation observed in the A264E mutant of P450 BM3 is an unprecedented heme iron ligand set. Spectroscopic studies indicate that it is present as a minor component of the substrate-free form of the A264E P450 BM3 enzyme, with the predominant form being the “normal” Cys-aqua ligated P450. However, binding of long chain fatty acids forces the equilibrium heavily in favour of the novel Cys-Glu ligand set, in similar fashion as binding of these substrates induces aqua ligand displacement and formation of a 5-coordinate high-spin heme iron species in the wild-type P450 BM3. An intriguing aspect evident from this study is that the A264E flavocytochrome P450 BM3 enzyme retains considerable fatty acid oxygenase activity, at least towards those fatty acids that are efficient in inducing the switch to Cys-Glu coordination. This indicates that the Glu ligand must be displaced (at least in a proportion of the enzyme) following the first reduction step in the catalytic cycle. This should allow oxygen to bind, reductive scission of dioxygen to occur following the second electron reduction of the iron, and for oxygenation of the tightly bound fatty acid substrate. In reporting their structure of the palmitoleate-bound form of the wild-type P450 BM3 heme domain, Li and Poulos noted that the distance between the ω-carbon of the bound substrate and the heme iron was too great for oxidative attack of the substrate, and that further structural change resulting in re-positioning of the substrate closer to the heme was required following reduction of the ferric iron (72). NMR studies of the ferric and ferrous P450 indicated that there should be a 6 Å movement of substrate subsequent to heme iron reduction (78). Thus, it appears clear that the Cys-Glu coordination is broken following heme iron reduction, enabling binding of oxygen and for catalysis to ensue, albeit with lower affinity than in the wild-type enzyme. Potentiometric analysis (Fig. 7) demonstrates that the reduction potential of the substrate-bound and substrate-free forms of the A264E heme domain are similar to one another (-315 ± 5 mV) and to that for the fatty acid-bound wild-type enzyme (-289 mV), consistent with the capacity of the reductase domain to mediate electron transfer to heme iron in the A264E flavocytochrome enzyme. The reduction potential of the substrate-free form of the wild-type P450 BM3 heme domain is -427 mV (37). The apparent near-identical heme iron reduction potentials of the substrate-free and arachidonate-bound forms of the A264E variant are
possibly a consequence of the same species undergoing reduction/oxidation in both cases. A likely scenario is that the Cys-aqua species is reduced preferentially (i.e. has a more positive reduction potential than the Cys-Glu species) and that the equilibrium between the Cys-aqua and Cys-Glu ferric forms is drawn towards the former as the reductive titration progresses. The altered heme iron reduction potential of the A264E mutant is discussed further in the accompanying paper, in light of major structural changes observed for the mutant.

In conclusion, the A264E variant of P450 BM3 produces an unprecedented heme iron ligand set (Cys-Fe-Glu), with the binding of long chain fatty acids forcing the equilibrium between the “native” Cys-aqua ligated form and the novel Cys-Glu ligated form towards the new ligation state. Structural reasons underlying this substrate-induced equilibrium perturbation are described in the accompanying manuscript. Differences in active site architecture between P450 BM3 and the mammalian CYP4 enzyme likely explain the failure of the BM3 A264E variant to covalently link the heme macrocycle to the glutamate sidechain. Such covalent attachment should still be feasible by altering the position of the introduced glutamate to avoid steric restrictions, or by combining A264E with secondary mutations. However, the results with the A264E variant open new avenues in the study of P450 heme coordination chemistry. Having created a completely novel Cys-Glu ligand set to the heme iron, the opportunity arises that other mutations at Ala264 could generate further sets of proteinaceous heme ligands that have not yet been observed in nature, possibly preempting the discovery of such cytochromes. This offers exciting possibilities for structural and spectroscopic study. In ongoing work we are generating a series of Ala264 mutants in P450 BM3 in attempts to produce and characterize further new heme iron coordination states.

**Acknowledgements**

AWM is grateful for the support from the BBSRC (UK) and EPSRC (UK) for this research. HMG is an EPSRC-funded PhD student. We are grateful to Prof. A. J. Thomson FRS and to Prof. D. J. Richardson for use of CMSB facilities at UEA.
REFERENCES

1) Munro, A.W. and Lindsay, J.G. (1996). Mol. Microbiol. 20, 1115-1125.
2) Guengerich, F.P. (1991). J. Biol. Chem. 266, 10019-10022.
3) Poulos, T.L., Finzel, B.C. and Howard, A.J. (1986). Biochemistry 25, 5314-5322.
4) Leys, D., Mowat, C.G., McLean, K.J., Richmond, A., Chapman, S.K., Walkinshaw, M.D. and Munro, A.W. (2003). J. Biol. Chem. 278, 5141-5147.
5) Wang, M., Roberts, D.L., Paschke, R., Shea, T.M., Masters, B.S.S. and Kim, J.-J. P. (1997) Proc. Natl. Acad. Sci. USA 94, 8411-8416.
6) Pochapsky, T.C., Jain, N.U., Kuti, M., Lyons, T.A. andHeymont, J. (1999). Biochemistry 38, 4681-4690.
7) Muller, J.J., Lapko, A., Bourenkov, G., Ruckpaul, K. and Heinemann, U. (2001). J. Biol. Chem. 276, 2786-2789.
8) Lee, D.S., Yamada, A., Sugimoto, H., Matsunaga, I., Ogura, H., Ichihara, K., Adachi, S., Park, S.Y. and Shiro, Y. (2003). J. Biol. Chem. 278, 9761-9767.
9) Munro, A.W., Leys, D.G., McLean, K.J., Marshall, K.R., Ost, T.W.B., Daff, S., Miles, C.S., Chapman, S.K., Lysek, D.A., Moser, C.C., Page, C.C. and Dutton, P.L. (2002). Trends Biochem. Sci. 27, 250-257.
10) Narhi, L.O. and Fulco, A.J. (1987). J. Biol. Chem. 262, 6683-6690.
11) Fulco, A.J. (1991). Annu. Rev. Phramacol. Toxicol. 31, 177-203.
12) Daff, S.N., Chapman, S.K., Turner, K.L., Holt, R.A., Govindaraj, S., Poulos, T.L. and Munro, A.W. (1997). Biochemistry 36, 13816-13823.
13) Sligar, S.G. and Gunsalus, I.C. (1976). Proc. Natl. Acad. Sci. USA 73, 1078-1082.
14) Groves, J.T. and Han, Y.-Z. (1995). Pp. 3-48 in “Cytochrome P450: Structure, Mechanism and Biochemistry”. Ed. Ortiz de Montellano, P.R. Published by Plenum Press (New York).
15) McLean, M.A., Maves, S.A., Weiss, K.E., Krepich, S. and Sligar, S.G. (1998). Biochem. Biophys. Res. Commun 252, 166-172.
16) Munro, A.W., Lindsay, J.G., Coggins, J.R., Kelly, S.M. and Price, N.C. (1996). Biochim. Biophys. Acta 1296, 127-137.
17) Hui Bon Hoa, G., McLean, M.A. and Sligar, S.G. (2002). Biochim. Biophys. Acta 1595, 297-308.
18) Perera, R., Sono, M., Sigman, J.A., Pfister, T.D., Lu, Y. and Dawson, J.H. (2003). Proc. Natl. Acad. Sci. USA 100, 3641-3646.
19) Modi, S., Primrose, W.U. and Roberts, G.C.K. (1995). Biochem. J. 310, 939-943.
20) Bell, S.G., Chen, X., Xu, F., Rao, Z. and Wong, L.L. (2003). Biochem. Soc. Trans. 31, 558-562.
21) Ost, T.W.B., Miles, C.S., Murdoch, J., Cheung, Y., Reid, G.A., Chapman, S.K. and Munro, A.W. (2000). FEBS Lett. 486, 173-177.
22) Glieder, A., Farinas, E.T. and Arnold, F.H. (2002). Nature Biotechnol. 11, 1135-1139.
23) Henne, K.R., Kunze, K.L., Zheng, Y.-M., Christmas, P., Soberman, R.J. and Rettie, A.E. (2001). Biochemistry 40, 12925-12931.
24) Hoch, U. and Ortiz de Montellano, P.R. (2001). J. Biol. Chem. 276, 11339-11346.
25) LeBrun, L., Hoch, U. and Ortiz de Montellano, P.R. (2002). J. Biol. Chem. 277, 12755-12761.
26) Zheng, Y.-M., Baer, B.R., Kneller, B., Henne, K.R., Kunze, K.L. and Rettie, A.E. (2003). Biochemistry 42, 4601-4606.
27) Oliver, C.F., Modi, S., Primrose, W.U., Lian, L.Y. and Roberts, G.C.K. (1997). Biochem. J. 327, 537-544.
28) Carmichael, A.B. and Wong, L.L. (2001). Eur. J. Biochem. 268, 3117-3125.
29) Ravichandran, K.G., Boddupalli, S.S., Hasemann, C.A., Peterson, J.A. and Deisenhofer, J. (1993). Science 261, 731-736.
30) Miles, J.S., Munro, A.W., Rospundowski, B.R., Smith, E.W., McKnight, J., Thomson, A.J. (1992). Biochem. J. 288, 503-509.
31) Noble, M.A., Miles, C.S., Chapman, S.K., Lysek, D.A., Mackay, A.C., Reid, G.A., Hanzlik, R.P. and Munro, A.W. (1999). Biochem. J. 339, 371-379.
32) Thomas, P.E., Ryan, D. and Levin, W. (1976). Anal. Biochem. 75, 168-176.
33) Mowat, C.G., Moysey, R., Miles, C.S., Leys, D., Doherty, M.K., Taylor, P., Walkinshaw, M.D., Reid, G.A. and Chapman, S.K. (2001). Biochemistry 40, 12292-12298.
34) Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual (2nd Edn.). Cold Spring Harbor Laboratory Press, New York.
35) Munro, A.W., Noble, M.A., Robledo, L., Daff, S.N. and Chapman, S.K. (2001). Biochemistry 40, 1956-1963.
36) Dutton, P.L. (1978). Methods Enzymol. 54, 411-435.
37) Ost, T.W.B., Miles, C.S., Munro, A.W., Murdoch, J., Reid, G.A. and Chapman, S.K. (2001). Biochemistry 40, 13421-13429.
38) Daff, S., Sharp, R.E., Short, D.M., Bell, C., White, P., Manson, F.D.C., Reid, G.A. and Chapman, S.K. (1996). Biochemistry 35, 6351-6357.
39) Thomson, A.J., Cheesman, M.R. and George, S.J. (1993). Methods Enzymol. 226, 199-232.
40) Munro, A.W., Daff, S., Coggins, J.R., Lindsay, J.G. and Chapman, S.K. (1996). Eur. J. Biochem. 239, 403-409.
41) Ruettinger, R.T. and Fulco, A.J. (1981). J. Biol. Chem. 256, 5728-5734.
42) Fulco, A.J. (1991). Annu. Rev. Pharmacol. Toxicol. 31, 177-203.
43) Buchanan, J.F. and Fulco, A.J. (1978). Biochem. Biophys. Res. Commun. 85, 1254-1260.
44) Narhi, L.O. and Fulco, A.J. (1986). J. Biol. Chem. 261, 7160-7169.
45) Macheroux, P., Massey, V., Thiele, D.J. and Volokita, M. (1991). Biochemistry 30, 4612-4619.
46) McKnight, J., Cheesman, M.R., Thomson, A.J., Miles, J.S. and Munro, A.W. (1993). Eur. J. Biochem. 213, 683-687.
47) Dawson, J.H., Andersson, L.A. and Sono, M. (1982). J. Biol. Chem. 257, 3606-3617.
48) Lipscomb, J.D. (1980). Biochemistry 19, 3590-3599.
49) Tsai, A.L., Berka, V., Chen, P.F. and Palmer, G. (1996). J. Biol. Chem. 271, 32563-32571.
50) Sono, M. and Dawson, J.H. (1982). J. Biol. Chem. 257, 5496-5502.
51) Sono, M., Hager, L.P. and Dawson, J.H. (1991). Biochim. Biophys. Acta 1078, 351-359.
52) Cheesman, M. R., Greenwood, C., and Thomson, A. J. (1991). Adv. Inorg. Chem. 36, 201-255.
53) Dawson, J.H., Andersson, L.A. and Sono, M. (1982). J. Biol. Chem. 257, 3606-3617.
54) Berka, V., Palmer, G., Chen, P.-F., and Tsai, A. L. (1998). Biochemistry 37, 6136-6144.
55) Shimizu, T., Nozawa, T., Hatano, M., Imai, Y., and Sato, R. (1975). Biochemistry 14, 4172-4178.
56) Vickery, L., Salmon, A., and Sauer, K. (1975). Biochim. Biophys. Acta 386, 87-98.
57) Dawson, J. H., Sono, M., and Hager, L.P. (1983). Inorg. Chim. Acta 79, 184-186.
58) Svastits, E. W., Alberta, J. A., Kim, I-C., and Dawson, J. H. (1989). Biochem. Biophys.
Res. Commun. 165, 1170-1176.
59) Sigman, J. A., Pond, A. E, Dawson, J. H., and Lu, Y. (1999). Biochemistry 38, 11122-
11129.
60) Rux, J. J., and Dawson, J. H. (1991). FEBS Lett. 290, 49-51.
61) Vickery, L., Nozawa, T., and Sauer, K. (1976). J. Am. Chem. Soc. 98, 351-357.
62) Berka, V., Palmer, G., Chen, P.-F., and Tsai, A. L. (1998). Biochemistry 37, 6136-6144.
63) Dhawan, I. K., Shelver, D., Thorsteinsson, D., Roberts, G. P., and Johnson, M. K. (1999).
Biochemistry 38, 12805-12813.
64) Gadsby, P. M. A., and Thomson, A. J. (1990). J. Am. Chem. Soc. 112, 5003-5011.
65) Cheesman, M.R., Little, P.J. and Berks, B.C. (2001). Biochemistry 40, 10562-10569.
66) Nozawa, T., Shimizu, T., Hatano, M., Shimada, S., Iizuka, T. and Ishimura, Y. (1978).
Biochim. Biophys. Acta 534, 285-294.
67) Munro, A.W., Lindsay, J.G., Coggins, J.R., Kelly, S.M. and Price, N.C. (1996). Biochim.
Biophys. Acta 1296, 127-137.
68) Modi, S., Primrose, W.U., Lian, L.Y. and Roberts, G.C.K. (1995). Biochem. J. 310, 939-
943.
69) Bell, S.G., Stevenson, J.A., Boyd, H.D., Campbell, S., Riddle, A.D., Orton, E.L. and
Wong, L.L. (2002). Chem. Commun. 7, 490-491.
70) Carmichael, A.B. and Wong, L.L. (2001). Eur. J. Biochem. 268, 3117-3125.
71) Joyce, M.G., Girvan, H.M., Munro, A.W. and Leys, D. (2004). J. Biol. Chem. (in press).
72) Li, H. and Poulos, T.L. (1997). Nat. Struct. Biol. 4, 140-146.
73) Oliver, C.F., Modi, S., Sutcliffe, M.J., Primrose, W.U., Lian, L.Y. and Roberts, G.C.K.
(1997). Biochemistry 36, 1567-1572.
74) Sutherland, J., Greenwood, C., Peterson, J. and Thomson, A.J. (1986). Biochem. J. 233,
893-898.
75) Cheesman, M.R., Thomson, A.J., Greenwood, C., Moore, G.R. and Kadir, F. (1990).
Nature 346, 771-773.
76) Arnesano, F., Banci, L., Bertini, I. and Felli, I.C. (1998). Biochemistry 37, 173-184.
77) Tsai, A.L., Berka, V., Chen, P.F. and Palmer, G. (1996). J. Biol. Chem. 271, 32563-32571.
78) Modi, S., Sutcliffe, M.J., Primrose, W.U., Lian, L.Y. and Roberts, G.C.K. (1996). Nat. Struct. Biol. 3, 414-417.
79) Noble, M.A., Quaroni, L., Chumanov, G.D., Turner, K.L., Chapman, S.K., Hanzlik, R.P. and Munro, A.W. (1998). Biochemistry 37, 15799-15807.
TABLES

Table 1
Comparative kinetic \( \frac{k_{\text{cat}}}{K_M} \) and substrate/inhibitor binding \( K_d \) constants for wild-type and A264E P450 BM3 enzymes. Binding constants were determined by optical titrations of fatty acids and heme ligands with the wild-type and A264E heme domain. Kinetic constants were derived from steady-state assays with the full-length wild-type and A264E flavocytochromes. All data were collected as described in the Experimental Procedures section. Data were fitted to rectangular hyperbolae, except in the cases of those data indicated by an asterisk, where data were fitted to equation 1. Selected values of \( \frac{k_{\text{cat}}}{K_M} \) and \( K_d \) for wild-type enzyme from previous studies are indicated as follows: \(^1\) from Noble et al. (1998) (reference 79); \(^2\) from Noble et al. (1999) (reference 31).
|                  | Wild-type BM3 | A264E BM3 |
|-----------------|--------------|-----------|
|                  | $K_d$ ($\mu$M) | $k_{cat}$ (min$^{-1}$) | $K_M$ ($\mu$M) | $K_d$ ($\mu$M) | $k_{cat}$ (min$^{-1}$) | $K_M$ ($\mu$M) |
| Laurate         | $100 \pm 15^1$ | $2770 \pm 120$ | $87 \pm 8$ | ND         | $744 \pm 21$ | $114 \pm 12$ |
| Myristate       | $6.9 \pm 0.4$  | $4835 \pm 295$ | $37.3 \pm 11.5$ | ND         | $1020 \pm 28$ | $10.9 \pm 1.3$ |
| Palmitate       | $11.3 \pm 0.4$ | $4590 \pm 407$ | $11.5 \pm 4.9$ | $0.045 \pm 0.021^*$ | $962 \pm 50$ | $16.4 \pm 3.6$ |
| Arachidonate    | $3.5 \pm 0.17$ | $17100 \pm 190^2$ | $4.7 \pm 0.25^2$ | $0.154 \pm 0.040^*$ | $1710 \pm 120$ | $0.65 \pm 0.15$ |
| Palmitoleate    | $0.55 \pm 0.05^*$ | $6980 \pm 430$ | $0.75 \pm 0.25$ | $0.214 \pm 0.019^*$ | $3090 \pm 90$ | $1.7 \pm 0.2$ |
| Cyt c           |             | $11050 \pm 630$ | $16.1 \pm 4.1$ |             | $15785 \pm 890$ | $28.7 \pm 6.5$ |
| 4-phenyl-imidazole | $0.85 \pm 0.45^*$ |          |          | $2.5 \pm 0.5^*$ |          |          |

Table 1
FIGURES

Figure 1
Alignment of P450 amino acid sequences in the region of the heme-ligating glutamate in CYP4 family
An alignment of amino acid sequences from various members of the CYP4 family of fatty acid oxygenating P450s was performed using the ClustalW sequence alignment program via the European Bioinformatics Institute web site at http://www.ebi.ac.uk/clustalw/. The sequence alignment shown is for residues in the I helix region of the P450s, surrounding the conserved glutamate residue shown to participate in covalent linkage of the heme group in eukaryotic CYP4 enzymes (23, 24). The relevant amino acids at this position in the alignment are underlined in bold. The amino acid sequences shown are for flavocytochrome P450 BM3 from Bacillus megaterium (CYP102A1) and its homologues in Bacillus subtilis (CYP102A2 and CYP102A3), rabbit CYP4A4, rat CYP4A1, human CYP4B1, cockroach CYP4C1, CYP4D1 from Drosophila melanogaster, and Pseudomonas putida P450 cam (CYP101).

Figure 2
Absorption properties of the A264E heme domain of flavocytochrome P450 BM3
Panel A shows the spectra for oxidised A264E heme domain (7 µM, thin solid line) and for the protein following addition of excess sodium dithionite reductant (dotted line) and formation of the carbon monoxy complex (thick solid line). The inset shows the difference spectrum generated by subtraction of the spectrum for the reduced form from that of the CO-complex. Panel B shows the spectrum of oxidised A264E heme domain (5 µM, thin solid line) and for the protein following saturation with the azole ligand 4-phenylimidazole (thick solid line) and following complexation with nitric oxide (dotted line).

Figure 3
Optical transitions associated with binding of arachidonic acid to the A264E heme domain
Panel A: Absolute spectra showing substrate-free A264E heme domain (thin, solid black line with Soret maximum at 420.5nm) with successive spectra recorded following addition of 1.65,
Novel heme ligation in a cytochrome P450

2.97 and 4.95 µM arachidonic acid (dotted lines) showing progressive deviation from the starting spectrum. The final spectrum shown was recorded at 8.25 µM arachidonic acid (thick, solid black line) with Soret maximum at 426 nm. The inset shows nested difference spectra from the arachidonic acid titration of the A264E heme domain, generated by subtraction of the starting absolute spectrum for fatty acid-free enzyme from those produced at subsequent points in the titration. Selected difference spectra (with progressive deviation from the baseline) are shown for data recorded at 0.66 µM, 0.99 µM, 1.32 µM, 1.65 µM, 1.98 µM, 2.31 µM, 2.97 µM, 3.3 µM, 3.96 µM, 5.28 µM, 5.61 µM, 6.6 µM and 6.93 µM. An isosbestic point is located at ~ 424.5 nm.

Panel B shows a plot of the arachidonic acid-induced spectral absorption change (A_{433} minus A_{415}) from the difference spectra versus [arachidonic acid]. The data are fitted to equation 1, yielding a K_d of 0.29 ± 0.05 µM.

Figure 4
Near UV-visible CD spectra for wild-type and A264E P450 BM3 heme domains in presence and absence of fatty acid substrate
Spectra were recorded for the wild-type P450 BM3 heme domain (20 µM) and for the A264E heme domain (20 µM), as described in the Experimental Procedures. Following spectral acquisition, arachidonic acid (100 µM) was added to both wild-type and A264E proteins and spectra were again recorded. All spectra show negative ellipticity in the region of the heme Soret band, with minima located at 409 nm for the substrate-free wild-type P450 (dashed line), shifting to 404 nm in the arachidonate-bound form (dotted line); and at 422 nm for the substrate-free A264E heme domain (thin, solid line), shifting to 426 nm for the arachidonate-bound form (thick, solid line).

Figure 5
EPR spectra for wild-type and A264E P450 BM3 heme domains in substrate-free, arachidonate-bound and 4-phenylimidazole-bound forms
Spectra were recorded for the wild-type P450 BM3 heme domain and for the A264E heme domain, as described in the Experimental Procedures. Fig. 5A: EPR spectra are shown for the oxidised substrate-free forms of wild-type and A264E heme domains (labelled WT and A264E,
respectively), and following binding of arachidonate substrate (labelled WT+S and A264E+S, respectively). Relevant g-values are assigned in each spectrum. **Fig. 5B:** EPR spectra are shown for wild-type (WT) and A264E mutant (A264E) heme domains in their 4-phenylimidazole complexes. Relevant g-values are assigned in each spectrum.

**Figure 6**

**Magnetic Circular Dichroism studies of the A264E heme domain**

MCD spectra in the near-UV visible region (**Fig. 6A**) and the NIR region (**Fig. 6B**) are shown for both the heme domains of wild-type (WT) P450 BM3 and the A264E mutant. Spectra are distinguished by different forms of lines, as indicated in the figure insets. Protein concentrations used were: WT 300 µM (NIR region) and 30µM (near-UV visible); WT + substrate 550 µM and 45µM; A264E 690 µM and 33µM; A264E + substrate 660 µM and 63µM.

**Figure 7**

**Redox potentiometric analysis of the A264E P450 BM3 heme domain**

The midpoint reduction potential of the heme iron of the A264E heme domain was determined in the presence and absence of arachidonic acid, as described in the Experimental Procedures section. **Fig. 7A:** Main figure shows selected spectra from the titration of the arachidonate-bound form of the A264E mutant. Arrows indicate direction of spectral change at selected points during the reductive phase of the titration. The spectral maximum shifts from 426 nm to 410 nm on conversion between oxidised (ferric) and reduced (ferrous) forms of the heme iron. Inset shows a plot of the proportion of oxidised enzyme (from A$_{410}$ data) *versus* applied potential, fitted to the Nernst function and producing a midpoint reduction potential of $-314 \pm 4$ mV for the heme iron. **Fig. 7B:** Main figure shows selected spectra from the titration of the substrate-free form of the A264E mutant. Arrows indicate direction of spectral change at selected points during the reductive phase of the titration. For clarity, fewer spectra are shown than in Fig. 6A, since in this case the spectral change on reduction occurs over a more compressed wavelength range (from ~419.5 nm to 410 nm). Inset shows a data fit (as in Fig. 6A), producing a heme iron midpoint reduction potential of $-316 \pm 3$ mV.
Figure 1

| CYP102A1 (B. megaterium) | 253 | IRYQIITFLIAGHETTSGLLSFLAYFLVK 282 |
| CYP102A2 (B. subtilis)  | 256 | IRFQIITFLIAGHETTSGLLSFATYFLLK 285 |
| CYP102A3 (B. subtilis)  | 256 | IRYQIITFLIAGHETTSGLLSFAIYCLLT 285 |
| CYP4A4 (Rabbit)         | 310 | LRAEVDVFMEGHDATSGVSMMLAT 339    |
| CYP4A1 (Rat)            | 309 | LRAEVDVFMEGHDATSGVSMMLAT 338    |
| CYP4B1 (Human)          | 304 | LRAEVDVFMEGHDATSGISWFNYALAT    |
| CYP4C1 (Cockroach)      | 303 | IREEVDFMEGHDATSSAGWCWALMLGS 332 |
| CYP4D1 (Drosophila)     | 305 | IREEVDFMEGHDATSSALMFNYNIAT 334  |
| CYP101 (P. putida)      | 238 | AKRMCGLLVGGLDTVNLSFSFRLAK 267  |
Figure 2
Figure 3
Figure 4
Figure 5A
Figure 5B
Figure 6
Figure 7
Flavocytochrome P450 BM3 mutant A264E undergoes substrate-dependent formation of a novel heme iron ligand set

Hazel M. Girvan, Ker R. Marshall, Rachel J. Lawson, David Leys, M. Gordon Joyce, John Clarkson, W. Ewen Smith, Myles R. Cheesman and Andrew W. Munro

J. Biol. Chem. published online March 12, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M401716200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts