Characterisation of flavocytochrome P450 BM3 site
directed mutants with novel heme ligation state

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Abstract

Uniquely among cytochromes P450, family 4 P450s have a conserved glutamate covalently attaching heme to the protein backbone. In the related *Bacillus megaterium* P450 BM3, the corresponding residue is alanine (A264). The relevant mutation (A264E) was constructed and characterised spectroscopically, kinetically and structurally. Heme is not covalently ligated in A264E BM3. Instead, E264 coordinates directly to heme iron, generating novel Glu/Cys heme axial ligation. E264 coordination is promoted by fatty acid substrate binding. EPR, MCD and resonance Raman showed that A264E remained low-spin with substrate bound, but retained limited catalytic activity. Both substrate-free and palmitoleate-bound A264E structures were solved and showed that one of two molecules in the substrate-free asymmetric unit had E264 ligation to heme iron, whilst the other had distal water. In all crystal forms, the structure was on a conformation only previously observed in substrate-bound wild-type enzyme. BM3 exists in a conformational equilibrium, and fatty acids bind preferentially to the "substrate-bound" conformation (proven by measurement of lower fatty acid $K_d$ values for A264E versus wild-type), rather than substrate inducing conformational change *per se*. In light of A264E's novel heme axial ligation state, other A264 mutants were generated. A264H and A264K mutants showed His/Cys and His/Lys ligation. Both mutants showed complete coordination by the amino acid side chains in presence or absence of substrates, and were inactive. A264Q and A264M mutants had limited catalytic activity and partial Gln/Cys or Met/Cys ligation, whilst A264C also showed limited activity, and partial Cys/Cys ligation from spectroscopic studies (but none detected in the crystal). Atomic structures were solved for all mutants and spectroscopic analysis provided first characterisations of novel heme iron ligand sets. Theses studies were complemented by analysis of reversible P450 conversion to and inactive (P420) form under high pressure, which was demonstrated to be caused by protonation of the proximal cysteinate.
## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>i</td>
</tr>
<tr>
<td>Contents</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>vi</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>vii</td>
</tr>
</tbody>
</table>

## Chapter 1  Introduction

1.1 *Heme containing proteins*  
1.2 *Cytochromes P450*  
1.3 *Redox Partners*  
1.3.1 Class I systems  
1.3.2 Flavin-Iron Sulphur-P450 fusions  
1.3.3 Diflavin reductases  
1.3.4 Flavodoxin-flavodoxin reductase utilising systems  
1.3.5 P450s that do not use redox partner proteins  
1.4 *Catalytic cycle*  
1.4.1 Substrate-free enzyme  
1.4.2 Substrate binding  
1.4.3 Introduction of first electron  
1.4.4 The Oxyferrous complex  
1.4.5 Introduction of the second electron and first proton  
1.4.6 Iron-peroxo species  
1.4.7 Uncoupling  
1.5 *P450 BM3*  
1.6 *Aims and objectives*  

## Chapter 2  Methods

2.1 *Molecular Biology*  
2.1.1 A264E Mutagenesis  
2.1.2 A264K/H/QM/C mutagenesis  
2.2 *Enzyme over-expression and purification*  
2.2.1 Overexpression  
2.2.2 Cell Harvesting and Lysis
2.2.3 Purification

2.3 SDS-PAGE

2.4 Protein concentration calculations

2.4.1 Reduced/CO adduct formation

2.4.2 Pyridine hemochromagen

2.5 Substrate and inhibitor binding studies

2.6 Steady state kinetics

2.7 Hydrogen peroxide and superoxide measurement

2.8 Redox potentiometry

2.9 Denaturation studies

2.9.1 Temperature effects on wild-type and mutant P450 BM3 heme domain spectral properties

2.9.2 pH effects on wild-type and mutant BM3 heme domain spectral properties

2.9.3 Ionic strength effects on wild-type and mutant BM3 heme domain spectral properties

2.9.4 Guanidine denaturation of A264E heme domain

2.10 Circular dichroism

2.11 Electron Paramagnetic Resonance (EPR)

2.12 Magnetic circular dichroism (MCD)

2.13 Resonance Raman

2.14 Product and substrate analysis

2.15 Crystallisation of A264E heme domain

2.16 Hydrostatic pressure spectroscopy

Chapter 3 Generation and characterisation of the A264E mutant of flavocytochrome P450 BM3 and its heme domain

3.1 Introduction

3.2 Results

3.2.1 A264E mutagenesis

3.2.2 Over-expression and purification of A264E BM3 enzymes

3.2.3 SDS-PAGE analysis

3.2.4 Comparisons of wild-type and A264E spectra

3.2.5 Substrate binding to wild-type P450 BM3 and A264E mutant heme domains

3.2.6 Inhibitor binding to A264E

3.2.7 Redox potentiometry
Chapter 4  Characterisation of A264K/Q/H mutant forms of flavocytochrome P450 BM3 and its heme domain: Novel P4S0 heme ligation states 138

4.1  Introduction 139

4.2  Results 144

4.2.1  Mutagenesis 144

4.2.2  Overexpression and purification of mutant BM3 enzymes 146

4.2.3  Calculation of heme concentration 147

4.2.4  Comparison of wild-type and A264Q/K/H mutant UV-visible spectra 148

4.2.5  Substrate-binding 150

4.2.6  Inhibitor binding 155

4.2.7  Effects of ionic strength, pH and temperature 158

4.2.8  Redox Potentiometry 162

4.2.9  EPR 168

4.2.10  MCD 169

4.2.11  Resonance Raman 175

4.2.12  Steady-state kinetic analysis 180

4.2.13  Product analysis 184

4.2.14  Measurement of uncoupled enzyme turnover 187

4.2.15  X-ray crystallography 189

4.3  Discussion 196

Chapter 5  Characterisation of A264C/M mutant forms of flavocytochrome P450 BM3 and its heme domain: Novel P4S0 heme ligation states 199

5.1  Introduction 200

5.2  Results 204

5.2.1  Mutagenesis 204

5.2.2  Overexpression and purification of A264M/C mutants 206

5.2.3  Calculation of heme concentration 207
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.2.4</td>
<td>Comparison of wild-type and A264M/C mutant UV-visible spectra</td>
<td>208</td>
</tr>
<tr>
<td>5.2.5</td>
<td>Substrate binding</td>
<td>210</td>
</tr>
<tr>
<td>5.2.6</td>
<td>Inhibitor binding</td>
<td>214</td>
</tr>
<tr>
<td>5.2.7</td>
<td>Redox Potentiometry</td>
<td>219</td>
</tr>
<tr>
<td>5.2.8</td>
<td>EPR</td>
<td>225</td>
</tr>
<tr>
<td>5.2.9</td>
<td>MCD</td>
<td>228</td>
</tr>
<tr>
<td>5.2.10</td>
<td>Resonance Raman</td>
<td>231</td>
</tr>
<tr>
<td>5.2.11</td>
<td>Steady-state kinetics</td>
<td>238</td>
</tr>
<tr>
<td>5.2.12</td>
<td>Product formation</td>
<td>240</td>
</tr>
<tr>
<td>5.2.13</td>
<td>Uncoupled turnover</td>
<td>242</td>
</tr>
<tr>
<td>5.2.14</td>
<td>Structural studies</td>
<td>243</td>
</tr>
</tbody>
</table>

| 5.3     | Discussion | 249 |

**Chapter 6**  Characterisation of novel P450 heme ligation states under extremes of pressure 251

| 6.1     | Introduction | 252 |
| 6.2     | Results | 254 |
| 6.3     | Discussion | 271 |

**Chapter 7**  Conclusions and future perspectives 274

**References** 281
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### Abbreviations

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Abbreviation</th>
<th>Code</th>
</tr>
</thead>
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<tr>
<td>AMP</td>
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<tr>
<td>PCR</td>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
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<td>Superoxide dismutase</td>
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</tr>
<tr>
<td>TMBZ</td>
<td>3,3',5,5'-tetramethylbenzidine</td>
<td></td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
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</tr>
</tbody>
</table>
Chapter 1  Introduction
1.1 Heme containing proteins

Heme is present as cofactor in a large number of enzymes, which catalyse a variety of reactions including electron transfer, oxygen transfer and monooxygenation. The heme moiety is the catalytic centre of each of these reactions, with a central iron atom, which is fundamental to the role of the protein or enzyme. The heme group varies greatly from protein to protein, both in the structure of the heme moiety and in the axial heme ligation. A number of different heme groups are shown overleaf in Figure 1.1.
Chapter 1 Introduction

Figure 1.1 Structure of hemes a, b, c and d
The structures of a number of biologically relevant hemes are shown, with the type of heme indicated below the structure in green. The pyrrole rings and their side chains are labelled on heme a, the same numbering applies to all other heme groups. Adapted from (1)

In addition to the variety of heme groups present in the cytochrome and globin proteins there is also a large amount of variety in the axial ligands to the heme iron. A number of examples is shown overleaf in Figure 1.2.
Figure 1.2  **Heme iron axial ligations**

A number of heme axial ligations found in nature are shown. His/His is one of the most common ligation states found, predominantly with both histidines ligated to the heme iron through their ε-nitrogen, but also with ligation through the δ-nitrogen. His/Met, His/Tyr (where R1 represents the tyrosine phenolic with axial ligation coming from the α amino group) and Met/Met ligations are other examples of hexacoordinate axial ligation states. A number of pentacoordinate axial ligation states are also found, including His, Tyr and Cys. Figure generated with ISIS draw and adapted from (1). The ligation states shown are discussed in more detail below.

As is demonstrated in Figure 1.1 and Figure 1.2, there are large variations in both the structural composition of the heme group and in the nature of the axial ligation to the heme iron. Together these variations have a significant effect on the properties of the protein (e.g. redox potential of the heme, ability to bind ligands etc) and are responsible for a large element of the protein’s ability to carry out its specific function.

Heme a is only found naturally in the multi-redox centre enzyme cytochrome c oxidase, which is the terminal enzyme of the mitochondrial respiratory chain (2,3).
Chapter 1 Introduction

Cytochrome \textit{c} oxidase is an integral membrane protein, which catalyses the translocation of four protons across the inner mitochondrial membrane with concomitant four electron reduction of dioxygen to water \cite{4}. The cofactors contained within cytochrome \textit{c} oxidase are a bimetallic copper centre, \textit{Cu}_{\text{A}}, a low-spin heme \textit{a} group with His/His ligation, and a bimetallic site composed of an iron from a second \textit{a}-type heme, named heme \textit{a}_3 and a second copper centre, \textit{Cu}_{\text{B}}. Heme \textit{a}_3 is high-spin pentacoordinate with proximal histidine ligation and a binding site for gaseous ligands \textit{i.e.} oxygen in order to fulfil its biological role) on the distal side \cite{5}. Heme \textit{a} is synthesised from the simpler precursor heme \textit{b} by the enzymes heme \textit{o} synthase and heme \textit{a} synthase \cite{6}, rendering it more hydrophobic than heme \textit{b} \cite{7}.

Heme \textit{b} is found in a large variety of proteins, including catalases, cytochromes \textit{b}, globins, cytochromes P450 and peroxidases. The catalase enzymes catalyse the formation of two water molecules and one dioxygen from two hydrogen peroxide molecules. They are also able to use peroxide to oxidise other molecules, again producing water as a by product \cite{8}. They generally contain a tyrosine ligated heme \textit{b}, although in a small number of cases the heme is modified to heme \textit{d} \cite{9}. The globin enzymes (e.g. myoglobin and haemoglobin) play an important role in oxygen storage and transport, each containing a proximal histidine ligand \cite{10}. The peroxidases utilise hydrogen peroxide as electron acceptor to catalyse a variety of reactions. Many peroxidases contain heme \textit{b} with histidine proximal ligand and no distal ligation, allowing binding of hydrogen peroxide \cite{11}. The cytochromes P450 generally catalyse the monooxygenation of substrate by introduction of an oxygen atom from molecular oxygen. They have cysteine proximal ligation and water as the distal ligand. Substrate binding displaces the aqua ligand and enables dioxygen binding. The cytochromes P450 are discussed in greater detail in subsequent sections.

Heme \textit{c} is found in the cytochromes \textit{c}, which are a large family of electron transfer proteins. Each cytochrome \textit{c} typically has its heme covalently attached to the protein polypeptide by 2 cysteine residues in the consensus sequence CXXCH, where the histidine residue forms the proximal heme iron ligation \cite{12}. The cytochromes \textit{c} play a number of vital roles in nature. One of the most important is as electron donor to cytochrome \textit{c} oxidase, which was discussed above. Electrons are delivered from the inner-membrane cytochrome \textit{bc}_1, which is complex III of the respiratory chain, to
cytochrome c that is found in the intermembrane space. Cytochrome c then passes the
electrons to the Cu₄ centre of cytochrome c oxidase (13). A second important role for
cytochrome c is in apoptosis. Under apoptotic stimuli cytochrome c is released from
the mitochondria and becomes one of the factors that induces proteolytic processing
and activation of the cell death protease caspase-3 (14). In addition to the histidine
proximal ligand, most cytochromes c also contain a distal ligand, often histidine or
methionine (12). As yet no role has been discovered for the covalent heme ligation
found in cytochromes c, although the linkages do affect the proximal histidine residue
altering the thermodynamic properties of the enzyme (15). It is also known that
accessory lyase proteins are required for proper folding and heme incorporation, with
prokaryotes, chloroplasts and mitochondria each having a different system (16). The
simple bis-His axially ligated cytochromes $b_5$ and $b_{562}$ have been mutated, changing a
surface asparagine to cysteine. These mutations resulted in a proportion of the enzyme
having covalent heme ligation, with ligation between the cysteine and a heme vinyl
group (17,18). This suggests strongly that the reaction is autocatalytic and relies
chiefly on appropriate positioning of the cysteine(s) with respect to the heme vinyl
groups.

Also shown in Figure 1.1 is heme $d_1$, which has only been found to date in cd$_1$ nitrite
reductase. It contains one molecule each of heme c and heme $d_1$ in each monomer, and
catalyses the reduction of nitrite to nitric oxide with the concomitant formation of
water (19). Electrons are transferred into the cytochrome c, whilst the heme $d_1$ is the
site of nitrite reduction. Heme $d_1$ is bound axially by the phenolic oxygen atom of a
tyrosine and a histidine, but on reduction loses the tyrosine ligand becoming 5-
coordinate and thus allowing substrate to bind (20). Overall, the heme $d_1$ porphyrin
ring is more saturated (reduced by 4 electrons more) than heme $b$ (21). The effects of
this are poorly understood, but the heme ring is more acidic than heme $b$ and the
reduction potential of the iron in the heme $d$ is increased by around 200mV compared
to that in heme $b$ (22).

The variety of heme groups and axial ligands illustrates the complexity of heme-
containing proteins and enzymes. Relatively little is known about the reasons for these
differing heme groups. However, the variation seen in the axial ligands generally
results in differing electron withdrawing or donating properties and thus plays a
fundamental role in the catalytic activity of the enzyme. For example the globins are 5-coordinate with a distal histidine ligand, the presence of which is thought to stabilise the binding of oxygen (23). The peroxidases also often contain a proximal histidine, with no distal ligand. In these enzymes the distal histidine plays 2 roles, initially stabilising the binding of peroxide, then enabling transfer of a proton to the distally bound oxygen. This, in conjunction with an arginine residue located near the histidine, results in oxygen bond cleavage and release of water. The histidine and arginine residues are described as the “pull effect” (24). As will be discussed in more detail in subsequent sections, the thiolate proximal ligation to the P450 heme iron is fundamental for splitting of molecular oxygen, providing the necessary electronic “push” for the reaction.

1.2 Cytochromes P450

Cytochromes P450 (P450s) are a family of heme \(b\) containing electron transfer enzymes. In the mid 1950s an enzyme system was discovered in the liver endoplasmic reticulum, by Axelrod (25) and Brodie et al. (26), which was shown to be capable of oxidising certain xenobiotic compounds. In the following years monooxygenase activity was found in liver microsomal preparations by Mason et al. (27). Garfinkel (28) and Klingenberg (29) separately showed that a pigment isolated from liver microsomal preparations was capable of binding carbon monoxide, giving an absorption maximum at 450nm. In 1964 Omura and Sato then showed that the pigment responsible for the absorption was a heme \(b\)-containing protein and coined the name cytochrome P450 (30,31). From this point there has been a huge amount of interest in the cytochromes P450, with over 3000 discovered to date in all classes of biota (32). This number is rapidly expanding with the completion of sequencing projects, for example the completion of the Arabidopsis sequencing project revealed the presence of 273 P450s and the rice genome revealed up to 400 (33).

The cytochromes P450 each contain an axial sulphur ligand from a cysteine contained in the polypeptide chain (34-36); this was confirmed with the solving of the first P450 structure (37). The cysteine is one of only a handful of residues that is implicitly conserved across the entire P450 superfamily.
Chapter 1 Introduction

The P450s generally, although not exclusively, catalyse the monooxygenation of an organic substrate bound close to the distal side of the heme iron. Molecular oxygen is the source of the oxygen added to the substrate. The other atom of oxygen from dioxygen is reduced to water. The reaction requires two electrons and these are delivered ultimately from NAD(P)H and almost invariably via one or more redox partner enzymes. An overview of this mechanism is shown in Figure 1.3 (38).

\[
\text{NAD(P)H} + \text{O}_2 + \text{SH} + 2\text{H}^+ \rightarrow \text{NAD(P)}^+ + \text{H}_2\text{O} + \text{SOH}
\]

**Figure 1.3** Overall reaction catalysed by cytochrome P450 enzymes.
NAD(P)H acts as electron donor, with P450s catalysing cleavage of molecular oxygen (O\(_2\)), forming hydroxylated substrate (SOH) and also producing oxidised NAD(P)\(^+\) and one water molecule.

A nomenclature system has been devised by Dr David Nelson to categorize the P450s on the basis of function and level of sequence identity. It is thought that the cytochromes P450 have a common ancestral form, with the first major evolutionary division coming between prokaryotic and eukaryotic forms, and subsequent divergence occurring from this point. Currently 154 bacterial families have been discovered, with 76 families in lower eukaryotes, 62 in plants, and 76 in animals (33). The P450s play important roles in the metabolism of both xenobiotic compounds and endogenous compounds. For example, P450s are involved in the conversion of cholesterol to progesterone in eukaryotes, and in testosterone and aldosterone metabolism in higher organisms. Large numbers of P450s are also involved in fatty acid metabolism and terpenoid metabolism. The exogenous compounds metabolised by the P450s include antibiotics like chloramphenicol, or drugs including isoniazid or paracetamol (39). The mammalian hepatic P450s have roles in xenobiotic/drug oxygenation, often leading to disposal of the molecule from the body. In many cases the products of these P450 reactions (phase I metabolism) are targeted for further conjugation or breakdown, ultimately facilitating excretion of the end products in urine or bile. However, in certain cases the P450s catalyse metabolic activation of procarcinogens or mutagens (27). A good example of this is their involvement in bioactivation of benzo-a-pyrene (39). The cytochrome P450 monooxygenases are capable of catalysing a variety of reactions, including hydroxylation, epoxidation and dealkylation. A number of examples is shown overleaf in Figure 1.4.
Chapter 1 Introduction

Figure 1.4 Examples of cytochrome P450 substrates and products

A huge variety of hydroxylations, epoxygenations, dealkylation and oxygenation reactions are catalysed by the cytochromes P450. A selection are shown above (40-45). The reactions catalysed are further discussed overleaf.
As was shown in Figure 1.4 a wide variety of reactions are catalysed by the cytochromes P450. A number of bacterial cytochromes P450 enable the bacterium to live in a particular habitat. For example P450 cam from *Pseudomonas putida* forms part of a pathway which allows the bacterium to utilise camphor as its sole carbon source (40). Similarly, the dealkylation of oxycoumarin by P450 RhF in *Rhodococcus* allows it to use the thiocarbamate as sole carbon source (46). P450 EryF catalyses the hydroxylation of deoxyerythronolide B in *Saccharopolyspora erythrae* forming part of the pathway for the biosynthesis of the antibiotic erythromycin (42). P450 BM3 from *Bacillus megaterium* catalyses the hydroxylation of fatty acids at \( \omega-1 \), \( \omega-2 \) and \( \omega-3 \) positions, but is less restricted than the other cytochromes P450 discussed previously in that it is capable of hydroxylating a large number of different fatty acids of differing chain length, degree of saturation and branch structure (47). Also shown in Figure 1.4 is the 14\( \alpha \)-demethylation of lanosterol catalysed by CYP51 (44). CYP51 is the only P450 family found to date which is present in all classes of biota, and is thought to be most ancient member of the cytochrome P450 family (48). In different species, the CYP51 isoforms which have been discovered show some variety in substrate with at least 4 different sterol substrates recognized as being physiologically significant substrates for CYP51 isoforms from various organisms. Each of these substrates (lanosterol, 24,25-dihydrolanosterol, 24-methylenedihydrolanosterol and obtusifoliol) is demethylated at the 14\( \alpha \) position for production of sterols that provide templates for further modification (via cholesterol) in the steroid biosynthesis pathway or for use (in yeasts) as cell membrane components (44). Finally, shown in Figure 1.4 are two of the reactions catalysed by cytochromes P450 with arachidonic acid as substrate. Arachidonic acid undergoes a wide number of reactions as substrate for the mammalian cytochromes P450. The CYP4A family are capable of \( \omega \)-hydroxylation of arachidonic acid which can act as a vasoconstrictor. The CYP2C family has been shown to epoxygenate arachidonic acid, which is then active as a vasodilator. Further modification of the products of arachidonic acid reaction with the cytochromes P450 can lead to prostaglandin formation (44). The substrates and products shown in Figure 1.4 are by no means an exhaustive list of the substrates utilised by the cytochromes P450, but demonstrate the diversity seen within the large enzyme superfamily.
Chapter 1 Introduction

1.3 Redox Partners

The catalytic cycle of the cytochromes P450 (P450s) requires that two electrons be delivered individually to the heme iron in the enzyme. These electrons are used to convert ferric heme iron to ferrous, allowing it to bind dioxygen, and to enable reductive activation of the bound dioxygen with cleavage of the O-O bond and insertion of an atom of oxygen into a substrate bound close to the heme iron. The electrons are generally, although not exclusively, derived from NADH or NADPH, and delivered to the heme via one or more redox partners (49). The major forms of P450 redox partners are discussed below.

1.3.1 Class I systems

The class I redox system utilises a flavin-containing ferredoxin reductase, usually with flavin adenine dinucleotide (FAD) as prosthetic group, and a ferredoxin with an iron-sulphur cluster as its redox centre. Ferredoxins containing different types of iron sulphur cluster (2Fe-2S, 3Fe-4S and 4Fe-4S) have been reported to support functions of various class I P450s. Class I systems are found in both eukaryotic mitochondria and bacteria (49).

The most studied examples of class I redox systems are putidaredoxin (Pdx) and putidaredoxin reductase (PdR) which shuttle electrons to the camphor hydroxylating enzyme P450 cam (the most studied of all cytochromes P450) from Pseudomonas putida allowing it to utilise camphor as its sole carbon source (50), and the mitochondrial adrenodoxin (Adx) and adrenodoxin reductase (AdR) which shuttle electrons to mammalian steroid hydroxylating P450s (51). PdR is a 46 KDa FAD-containing reductase (52) which receives reducing equivalents from NADH (53). It accepts 2 electrons from NADH as a hydride iron, reducing the FAD to its hydroquinone form. The first electron is passed to Pdx leaving a stable blue, neutral FAD semiquinone on PdR (54). Pdx then delivers the electron to the camphor-bound form of P450 cam and reoxidises. Thereafter, the second electron is passed to Pdx, returning the PdR to its original oxidised (quinine) form (55). Pdx contains an iron-sulphur cluster containing 2 iron atoms coordinated by two cysteine residues in the protein (a 2Fe-2S redox centre) (40,56) and acts as a shuttle between PdR and P450 cam transferring one electron at a time to P450 cam (50), as required by the P450
catalytic cycle, discussed below in section 1.4. The electron transfer mechanism in the AdR/Adx system employed by mammalian steroid hydroxylating cytochromes P450 differs little in its basic electron transfer mechanism, with the most notable difference being that NADPH supplies electrons to AdR, rather than NADH as in PdR (57).

![Diagram of electron transfer route from NAD(P)H to cytochrome P450](image)

**Figure 1.5** Electron transfer route from NAD(P)H to cytochrome P450 utilising the class I redox system
The class I redox system shuttles electrons from NAD(P)H to a FAD-containing ferredoxin reductase, initially forming the FAD hydroquinone. Electrons are then passed individually to the 2Fe-2S of ferredoxin which in turn passes the electron to the cytochrome P450 at different stages in its catalytic cycle. Only one ferredoxin is utilised by the class I redox system, passing through 2 oxidised-reduced-oxidised cycles, 2 are shown for diagrammatic simplicity and to illustrate that electrons enter the heme iron at different points in the P450 catalytic cycle. No specific details of the P450 cycle are shown in the scheme above. These are discussed in section 1.4
Adapted from Gunsalus et al. (38).

### 1.3.2 Flavin-Iron Sulphur-P450 fusions

Recently a number of P450s have been cloned from bacterial species in the genus *Rhodococcus*, with interest in this genus arising from their ability to utilise thiocarbamates as their sole carbon and nitrogen source, and therefore being capable of degrading herbicides (58). P450 RhF from *Rhodococcus sp* NCIMB 9784, has been found to comprise a novel redox partner fusion to the P450. Sequence alignments have revealed a C-terminal reductase domain, with a NADH-binding region, a FMN-binding region and a 2Fe-2S ferredoxin centre, predicted to be connected by a polypeptide linker to the P450 domain (46). Roberts *et al.* have cloned and recombinantly expressed the intact P450 RhF and have shown by whole cell
biotransformation that it is capable of the O-dealkylation of 7-ethoxycoumarin, a marker reaction for several eukaryotic P450s, at low levels. However, this artificial fluorogenic substrate is clearly not a mimic of the true physiological substrate.

![Reaction catalysed by P450 RhF.](image)

**Figure 1.6 Reaction catalysed by P450 RhF.**
The artificial substrate 7-ethoxycoumarin undergoes O-dealkylation forming 7-hydroxycoumarin. The reaction demonstrates that P450 RhF is functional as a catalytically self-sufficient NAD(P)H dependent P450 oxygenase. The reaction is distinct from any pathway that the P450 may participate in for utilisation of thiocarbamates as a sole carbon source in the host *Rhodococcus*.

Studies by Hunter et al. have further elucidated the thermodynamic properties of the P450 Rhf system, through analysis of the redox potentials of the redox active cofactors in the individually expressed domains of the system. They calculated the potential of the FMN sq-hq as -284 mV, FMN ox-sq as -232 mV and 2Fe2S as -214 mV. Their studies expand earlier work and suggest that the electron transfer pathway adopted by P450 Rhf is largely the same as that utilised by the class I cytochromes P450, with the obvious exceptions that the first redox centre is FMN rather than FAD as seen in the class I system, with electrons passing from NAD(P)H to the FMN group, through the iron sulphur centre and finally to the heme (59). Sequence analysis has also suggested that similar P450, iron-sulphur, flavin fusion enzymes also exist in a number of *Burkholderia* species and *Ralstonia metallidurans* (60).

### 1.3.3 Diflavin reductases
Eukaryotic endoplasmic reticulum P450s, a growing number of bacterial P450s and a number of other eukaryotic redox enzymes have been found to utilise a diflavin reductase as their redox partner e.g. (61-66). The diflavin reductases contain two
redox cofactors: one molecule each of FMN and FAD, both cofactors being non-covalently bound in a single polypeptide (67). Generally, eukaryotic P450s utilising a diflavin reductase as a redox partner are membrane bound (27), with the diflavin reductase also being an integral membrane protein tethered to the membrane by a hydrophobic N-terminal anchor domain (68,69). P450s utilising this system are categorised as class II. The second group of P450s utilising a diflavin reductase redox system are generally bacterial, with the P450 and diflavin reductase fused in a single polypeptide. These are often termed class III P450 systems (70) and the fusion protein is fully water-soluble and devoid of membrane anchors in either P450 or reductase domains. Membrane-bound, eukaryotic P450s have also now been found with their diflavin reductase and P450 contained in a single polypeptide (65). There are also a number of diflavin reductases that have contributed to our understanding of the structure and mechanism of this enzyme class, which do not pass electrons to a P450. A number of these are discussed below. Studies by Porter and Kasper have shown high levels of DNA sequence and amino acid homology exists between the FMN-binding domain of cytochrome P450 reductase (and other diflavin reductases) and bacterial flavodoxins. A high degree of similarity also exists between the FAD-binding domain of cytochrome P450 reductase and both ferredoxin-NADP+ reductase and NADH cytochrome b$_5$ reductase, leading them to suggest that the diflavin reductases have evolved from fusion between two different flavoproteins (71).

1.3.3.1 Class II diflavin reductases

Cytochrome P450 reductase is a diflavin reductase found bound to the endoplasmic reticulum and is responsible for the oxidation of NADPH and subsequent reduction of a P450 also fused to the membrane (68,69). This section will discuss mainly recent data from the study of human cytochrome P450 reductase (CPR). However, it is important to recognise that only the atomic structure of the rat CPR isoform has been solved (72), which shows a high level of sequence identity with the human isoform. In order to obtain crystals of the rat CPR, truncation of the intact enzyme was performed to remove the N-terminal membrane anchor.
1.3.3.2 Class III cytochromes P450s

The class III P450s are those with their P450 fused directly to the diflavin reductase in a single polypeptide. To date all P450s characterised belonging to this class are microbial, with the most studied example being the *Bacillus megaterium* fatty acid hydroxylase P450 BM3 (CYP102A1) (73). BM3 is discussed in more detail in section 1.5. Other P450s utilising a class III redox partner system have been discovered in recent years including CYP102A2 and CYP102A3 from *Bacillus subtilis*, which preferentially hydroxylate branched chain and unsaturated fatty-acids at the ω-1 position (74).

1.3.3.3 Eukaryotic membrane bound flavocytochromes P450

A membrane bound counterpart to P450 BM3 has been found in the fungus *Fusarium oxysporum*. The P450, named P450foxy, contains both P450 domain and CPR domain fused in a single polypeptide. P450foxy has 35.3% identity between its reductase domain and the reductase domain of BM3, while their P450 domains share 40.6% identity (75). It has also been found that P450foxy contains a N-terminal membrane anchor that links the protein to the yeast membrane via its P450 domain (65). It catalyses the ω-1, ω-2 and ω-3 hydroxylation of fatty acids, preferentially of C9 to C16 chain length with catalytic rates of 1200 to 1800 min⁻¹ (43), which are comparable with the rates catalysed by P450 BM3 with fatty acids of similar chain length (76).

1.3.3.4 Non-P450 diflavin reductase systems

A number of other diflavin reductases have been identified that serve to pass electrons to non-P450 redox partner enzymes. Many of these enzymes have been studied in detail in order to aid our understanding of the general electron transfer mechanism in this enzyme class and to characterise the nature of their interactions with their individual partner enzymes.

One obvious example of this type of enzyme is the family of eukaryotic nitric oxide synthases (NOS). Eukaryotic NOS enzymes are BM3-like fusions of a diflavin
Chapter 1 Introduction

reductase (C-terminal) and a heme b-containing oxygenase enzyme. Bacterial forms of NOS do not possess a fused diflavin reductase. The bound heme b is thiolate ligated, as in the P450s, and NOS performs P450-like reductive scission of dioxygen bound to the heme iron (77). Interest in NOS has arisen primarily from the physiological importance of the product nitric oxide. Nitric oxide has roles as a neurotransmitter, is involved in control of blood pressure and is a cytotoxic agent in macrophages (78).

The thiolate-ligated heme means that heme iron coordination in NOS is identical to that seen in the P450s, and NOS is sometimes classed alongside the cytochromes P450. However NOS also requires the protein calmodulin (CaM) and the additional cofactor tetrahydrobiopterin (H4B) for catalytic function, setting it apart from the P450s mechanistically (79). A second major difference is the lack of structural similarity between the oxygenase domain of NOS and the cytochromes P450. The P450s are largely alpha helical, whereas the NOS isoforms have a large component of beta sheet (80). Eukaryotic NOS is found in three forms neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS), each of which catalyse the conversion of L-arginine to L-citrulline and nitric oxide in two distinct oxygen-dependent steps (81).
Chapter 1 Introduction

Each of the NOS isoforms contains three domains, an N-terminal oxygenase domain which binds heme, $\text{H}_4\text{B}$, and arginine; a CaM-binding domain and a reductase (CPR-like) domain (83). CaM binding to nNOS and eNOS triggers electron transfer, (84,85), by affecting affinity for NADP(H) (86). CaM binds to NOS only in its calcium-bound form (87). The role of calmodulin in NOS catalysis indicates the importance of calcium as a signalling molecule in the NOS mechanism. Thus, for nNOS and eNOS, activity of the enzymes is subject to regulation by cellular levels of calcium ions. Unlike nNOS and eNOS, iNOS is not dependent on $\text{Ca}^{2+}$/CaM for activity, since the affinity is much higher and the CaM remains essentially completely bound at physiologically relevant calcium levels. Instead, iNOS appears to respond to increases in the immunological signalling molecule $\gamma$-interferon instead (88). In contrast to P450 BM3 and the other flavocytochrome enzymes, the eukaryotic NOS enzymes also require $\text{H}_4\text{B}$ for activity (89). It is now clear that $\text{H}_4\text{B}$ plays a role in transfer of an electron to the heme dioxygen intermediate facilitating product
formation. It is likely that $H_4B$ provides the second electron in at least the second catalytic step (transformation of L-hydroxyarginine to L-citrulline and nitric oxide) (90). The final major difference between the flavocytochromes P450 and the NOS enzymes is that the P450s usually catalyse a single round of oxygenation on a substrate (although e.g. P450 BM3 can perform successive rounds of hydroxylation on certain fatty acid substrates), whereas NOS enzymes catalyse two successive rounds of oxygenation of the substrate, using the product from the first round as the substrate for the second oxidative transformation. It is also important to note that one of the products of NOS (nitric oxide) is itself an inhibitor of NOS turnover, and the various NOS isoforms have had to evolve to optimise turnover rate in the presence of a product which has high affinity for both ferric and (particularly) ferrous forms of the enzymes (91).

A second example of a diflavin reductase which does not shuttle electrons to a cytochrome P450 is methionine synthase reductase (MSR). MSR plays a role in maintaining the activity of methionine synthase (MS). MS catalyses the transfer of a methyl group from methyltetrahydrofolate to homocysteine, forming methionine (92). Over time MS becomes inactivated when cob(I)alamin, an intermediate form of the MS redox active cofactor, becomes oxidised to cob(II)alamin (93). MSR catalyses the reductive methylation of cob(II)alamin with S-adenosylmethionine as a methyl donor (64,94). Homocysteine is a key risk factor in Down’s syndrome and expectant mothers are encouraged to take the nutrient folic acid during pregnancy. Folate is essential to provide the material required as the methyl donor for detoxification of the rogue metabolite homocysteine catalysed by MS (95). In turn, MSR is essential for regenerating the detoxification system.
Figure 1.8 Reactions catalysed by methionine synthase and methionine synthase reductase. Methionine synthase catalyses the transfer of a methyl group from methyltetrahydrofolate ($\text{CH}_3$-$\text{THF}$) to homocysteine, forming methionine. The reaction proceeds via the enzyme intermediate methionine synthase cob(I)alamin (MetSyn-CO(I)). The cob(I)alamin can become inactivated to cob(II)alamin, which methionine synthase reductase reactivates by catalysing its reductive methylation to cob(III)alamin (Met-Syn-CO(III)-$\text{CH}_3$) (64,96).

A third example of a diflavin reductase probably not involved in shuttling electrons to a P450 is novel reductase 1 (NR1) (61). As yet no physiological role has been found for NR1, but its slow catalytic rates, when compared to other diflavin reductases, has drawn interest to it (97). In addition, expression of NR1 is strongly up-regulated in several human cancer cell lines (97). The ability of NR1 to deliver electrons to methionine synthase has been demonstrated, although it is probably unlikely that this represents a physiologically relevant reaction (98). The slow rate of NR1 with respect to electron transfer to exogenous electron acceptors is due to the rate of hydride transfer to the FAD cofactor ($\approx 1 \text{ s}^{-1}$), which in turn appears due to the absence of key "catalytic triad" residues present in other diflavin reductases that promote binding of and hydride transfer from NADPH (97).
1.3.3.5 Diflavin reductase electron transfer mechanism

Detailed kinetic and thermodynamic studies of the different diflavin reductase enzymes has allowed elucidation of several aspects of their electron transfer mechanism. Redox potentiometry has been carried out on the isolated FAD-NADPH and FMN domains of each of the enzymes, giving an insight into the thermodynamic properties of the diflavin reductases. The redox potentials calculated are shown in Table 1.1.

<table>
<thead>
<tr>
<th></th>
<th>CPR (99)</th>
<th>BM3 (100)</th>
<th>NOS (101)</th>
<th>MSR (96)</th>
<th>NR1 (97)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAD_{ox/sq}</td>
<td>-286</td>
<td>-269</td>
<td>-232</td>
<td>-222</td>
<td>-315</td>
</tr>
<tr>
<td>FAD_{sq/hq}</td>
<td>-371</td>
<td>-337</td>
<td>-280</td>
<td>-288</td>
<td>-365</td>
</tr>
<tr>
<td>FMN_{ox/sq}</td>
<td>-43</td>
<td>-206</td>
<td>-49</td>
<td>-112</td>
<td>-152</td>
</tr>
<tr>
<td>FMN_{sq/hq}</td>
<td>-280</td>
<td>-177</td>
<td>-274</td>
<td>-221</td>
<td>-304</td>
</tr>
</tbody>
</table>

In the forms purified from expression systems, CPR, MSR and NR1 have an FMN cofactor in a neutral blue semiquinone form. This “air stable” semiquinone form takes several hours to reoxidise in air, and it has been hypothesised that the FMN resides “naturally” in this state in the cell. Hydride transfer to the 1-electron reduced form then forms a three electron reduced state, which then passes two electrons individually to their respective redox partners (first from the FMN hydroquinone and second probably from the FMN semiquinone), returning the system to the resting state with the FMN as a semiquinone following inter-flavin electron transfer from FAD semiquinone to the oxidised FMN (96,97,99). In each of the enzymes, the most negative couple is the FAD_{sq/hq} couple, and it may be the case that the enzymes do not populate this state to any significant extent during catalytic turnover. The redox potentials, and studies of the reverse reaction between the sodium dithionite-reduced FAD_{sq} and NADP⁺ have suggested that the FMN domains play an important role in ensuring electron transfer occurs in the forward direction (i.e. to FMN and then to the ultimate acceptor) and in preventing futile electron cycling between the FAD and
Chapter 1 Introduction

NADPH by removing one or both electrons to the FMN (96,97,99). Since the reduction potential of the NADP+/NADPH couple (-320 mV) is close to that of the FAD oxidised/hydroquinone in many of these enzymes, the back reaction to reduce NADP+ is thermodynamically feasible. Thus, there is a clear physiological advantage for the evolution of a fused high potential FMN-binding domain in the diflavin reductases. By contrast, the redox potentials of P450 BM3 suggest that the hydroquinone form of the FMN domain is more thermodynamically favourable than the semiquinone form, and that a neutral blue semiquinone form observed in potentiometric titrations resides on the FAD and not the FMN (100). Thus, this enzyme does not form an air stable FMN semiquinone and is purified in a fully oxidised state. This has also been shown using EPR spectroscopy (102) and inferred from UV-visible spectroscopy (103). Murataliev and Feyereisen used EPR of transiently reduced enzyme samples to demonstrate that the FMN in fact populates a transient FMN anionic (red) semiquinone (104). More recent studies on the FMN domain of P450 BM3 showed the UV-visible spectral characteristics of this FMN anionic semiquinone, and potentials for the two relevant couples were estimated as –240 ± 10 mV (FMN ox/sq) and –160 ± 10 (FMN sq/hq) (105). Thus, the semiquinone form of the FMN is thermodynamically destabilized in favour of the hydroquinone (105). P450 BM3 is isolated in a fully oxidised form (unlike human CPR) and the redox potentials therefore suggest that P450 BM3 cycles from the fully oxidised state through a two electron reduced state to a one electron reduced state and returning to the resting fully oxidised form (100). The cycle of electrons is thus 0-2-1-0 on the flavins. In fact, over-reduction of the flavins (i.e. addition of excess NADPH in the absence of fatty acid) produces an enzyme with diminished activity, that can be reactivated by reoxidation of the flavins, as discussed further below (106).

Stopped-flow and temperature-jump techniques have been used to study further electron transport within a number of the diflavin reductases. Gutierrez et al. (107) have shown that reduction of human CPR by the physiological reductant NADPH occurs in three distinct phases. In the first two phases charge transfer species are formed between the FAD and NADPH, and only in the third phase does hydride transfer occur, following which the enzyme forms an equilibrium with both flavins in the semiquinone form, involving inter-flavin electron transfer. Gutierrez et al. also showed that high concentrations of NADPH diminish the observed rate of hydride
transferred; leading the authors to hypothesise that there may be a second, inhibitory NADPH binding site on the enzyme (107). Investigation of inter-flavin electron transfer using temperature-jump techniques has shown that, in a 2-electron reduced species, electron transfer proceeds from the FAD$_{eq}$/FMN$_{eq}$ species to form a FAD$_{ox}$/FMNhq species when the temperature is elevated by ~7 °C on a microsecond timescale (108). T-jump indicates that this inter-flavin electron transfer occurs several-fold slower than is predicted on the basis of the distance between the two flavin when dithionite is used as electron donor(< 4 Å from the crystal structure), at only 11 s$^{-1}$. The fact that this rate depends on the nature of the reductant (dithionite or NAD(P)H – electron transfer being faster when the natural pyridine nucleotide reductant is used) and that the rate is accelerated in dithionite-reduced CPR by the addition of 2' AMP suggests strongly that electron transfer is "gated" by bound pyridine nucleotide cofactor (108).

P450 BM3 differs from other diflavin reductases in that it is isolated as a fully oxidised enzyme. On reduction with excess NADPH a blue semiquinone develops. However, as noted above, this semiquinone is located on the FAD rather than on the FMN. Studies of the electron transfer mechanism have shown that hydride transfer from NADPH to the FAD cofactor can be reversed, with FADH$_2$ capable of passing electrons back to NADP$^+$ in the form of a hydride ion (104). On the basis of thermodynamic considerations (potential for NADP$^+$/NADPH = -320 mV; potential for FAD ox/hq = -303 mV) it is not altogether surprising that the reaction is reversible. These data again suggest that the FMN has a role in preventing futile cycling of electrons between the FAD and NADPH and ensuring direction of electron flow is towards the heme iron, as was suggested for rat and human CPR. It has also been shown that following reduction by NADPH under catalytic conditions, the disemquinone species is rapidly formed (109). As noted above, using EPR Murataliev and Feyereisen found that both red (anionic) semiquinone and blue (neutral) semiquinone signals were presented when the overall EPR data were deconvoluted indicated the presence of roughly equimolar quantities of the two semiquinones (104). Since studies of individual domains had demonstrated that the FAD formed a neutral semiquinone, it was concluded that the FMN formed the anionic species. Recent work on the spectral properties of the reduced FMN domain of P450 BM3 support this conclusion (105). In early work, Fulco's group showed that pre-reduction of the enzyme with NADPH resulted in
formation of a 3-electron reduced form that was kinetically very slow as a fatty acid hydroxylase following the subsequent addition of fatty acid substrate. However, the enzyme retained normal levels of cytochrome c reductase activity. Reoxidation of the flavins restored the enzyme to its “active” fatty acid hydroxylase form (62). It is necessary for the enzyme to be in the fully oxidised state for efficient lipid oxygenation and NADPH oxidation to proceed (109). This may be due to conformational changes associated with over-reduction of the enzyme, or to sluggish electron transfer from the FMN$_{0q}$ to the heme iron (i.e. electron transfer should be from the FMN$_{sq}$ in the 0-2-1-0 model). Recent studies of the enzyme indicate that the dimeric form of the enzyme is catalytically active in fatty acid hydroxylation – but that the monomeric form is not (110). Thus, redox state may potentially affect the monomer/dimer equilibrium – although further work is required to ratify this hypothesis. Taken together, studies of electron transfer within the diflavin reductase of BM3 suggest that its mechanism proceeds as is shown in Figure 1.9 below.

\[
\text{NADPH} + \text{FAD}_{\text{ox}} \text{FMN}_{\text{ox}} \rightleftharpoons \text{NADP}^+ + \text{FAD}_{\text{sq}} \text{FMN}_{\text{sq}} \rightarrow \text{FAD}_{\text{sq}} \text{FMN}_{\text{sq}}
\]

**Figure 1.9 Flavocytochrome P450 BM3 diflavin reductase electron transfer mechanism.**
Electron transfer proceeds from NADPH, transiently forming the FAD hydroquinone, before the disemiquinone is formed, and the first electron is passed to the P450 by the FMN. The second electron is then passed to FMN, and again to the P450, returning the diflavin reductase to the fully oxidised form (100,103,104,109,111).

Studies of electron transfer have also been carried out with the cancer-related diflavin reductase NR1 by Finn *et al*. NR1 FAD reduction by NADPH occurs around 200 fold slower than that seen with CPR, at ~ 1 s$^{-1}$. The authors have suggested that the reason for this is the presence of an alanine residue (Ala 549), which in other diflavin reductases is a cysteine that forms part of a catalytic triad important for binding of NADPH and enabling electron transfer to the flavin (97). In addition, an aspartic acid
in the triad is also replaced by a glutamic acid (Glu 594) in NR1. The catalytic triad of P450 BM3 has also been studied. In BM3 the triad consists of cysteine 999, serine 830 and aspartate 1044. In NR1, the lack of the cysteine in the catalytic triad was hypothesised to be as a major determinant for the relatively slow rate of hydride transfer from NADPH to the FAD in the enzyme (97). This hypothesis has been supported by Roitel et al. in studies of BM3, who have shown severe retardation of the rate of hydride transfer in BM3 when Cys 999 is replaced with an alanine (112). However, the reason for the replacement of the triad residues in NR1 is not clear, and perhaps will only be resolved when the natural partner enzymes for this reductase are defined.

Although BM3 appears to have differences in electron transfer mechanism compared with certain other members of the diflavin reductase family (resulting mainly from the structural and thermodynamic properties of the FMN domain), it is still highly related to the other diflavin reductase enzymes, therefore ensuring that it remains a viable model system for the study of its mammalian counterparts.

1.3.4 Flavodoxin-flavodoxin reductase utilising systems
As stated above, class I-type P450 redox systems that appear to use a flavodoxin (rather than a ferredoxin) are now being discovered from various bacteria. P450cin (CYP176A) has recently been isolated from Citrobacter braakii, cloned, expressed and partially characterised. P450cin is believed to be involved in the initial monooxygenation of cineole 1, a monoterpene produced by eucalyptus plants (113). During sequence analysis it was discovered that two genes directly upstream encode a putative FAD-dependent flavodoxin reductase, and a FMN-containing flavodoxin. The operons containing the genes for P450 cam and P450 terp, both of which are also monoterpenic oxygenases, have their redox partners located directly upstream, possibly to aid tighter regulation of expression (50,114). This supports the hypothesis that the flavodoxin reductase and flavodoxin are the redox partners for P450cin (113). The structure of P450cin in complex with cineole has been solved by X-ray crystallography (115) and presents a number of interesting features, including the absence of certain amino acids which are highly conserved in other P450s. – This feature is discussed in
more detail in section 1.5.1. The structure also shows a strongly negatively charged distal face and slightly positively charged proximal face, suggesting a redox partner docking region (115) as is seen in P450 cam (116) and P450 BM3 (117). These data again support the hypothesis that the enzyme utilises a flavodoxin and flavodoxin reductase as redox partner, with charge complementarity enabling docking of the flavodoxin on the proximal face of the P450. This appears to be the first characterised system in which a P450 utilises a host flavodoxin as the redox partner between flavodoxin reductase and the P450 heme. However there are obvious analogies with the CPR-utilising P450 systems – since the FMN domains of CPRs structurally resemble the flavodoxins. Recently, host flavodoxins were also shown to support the catalytic function of the P450 BioI (CYP107H1) from Bacillus subtilis. This P450 is involved in the pathway for synthesis of the vitamin biotin (118).

1.3.5 P450s that do not use redox partner proteins

A number of cytochromes P450 function in differing manners to those discussed above. As discussed briefly below, some do not require a redox partner protein for donation of electrons, and others utilise electron donors other than reduced pyridine nucleotide coenzymes.

1.3.5.1 P450nor

P450nor was first identified in the fungus Fusarium oxysporum under induction from nitrite or nitrate (119), and was subsequently identified in a number of other fungi (120-122). Unlike almost all other cytochromes P450, P450nor does not catalyse the monooxygenation of a substrate involving reductive scission of molecular oxygen. Instead, P450nor catalyses the reduction of two molecules of nitric oxide (NO) to nitrous oxide (N₂O) with simultaneous production of a molecule of water, as is shown in Figure 1.10 (123,124).
Chapter 1 Introduction

\[ 2\text{NO} + \text{NAD(P)H} + \text{H}^+ \rightarrow \text{N}_2\text{O} + \text{NAD(P)}^+ + \text{H}_2\text{O} \]

**Figure 1.10** Overall reaction catalysed by P450nor.

Nitric oxide is converted to N\(_2\)O with NAD(P)H as a hydride donor, one proton is consumed in the reaction and one water molecule produced.

There is no requirement for a redox partner to facilitate the reaction catalysed by P450nor, with the *F. oxysporum* enzyme utilising NAD(P)H directly as hydride donor (123), and capable of achieving high rates of enzyme turnover (125). The crystal structure of P450nor from *F. oxysporum* has been solved, and revealed that the enzyme has fundamentally the same structure as the other structurally characterised P450s (126). The principal difference with the structure is the heme-distal pocket, which contains a wide, hydrophobic opening composed of a large number of positively charged lysine and arginine residues, as is shown in Figure 1.11. These residues are not present in the other cytochromes P450 for which structures are available. A charge neutralising mutagenic study has confirmed the importance of this region of the enzyme for NADH binding (127), and the structure has also been elucidated with NADH bound, confirming that the enzyme is capable of binding NADH directly and allowing identification of putative hydride access channels (128).
Comparison between P450 BM3 and P450nor structures

Structures A and B show P450 BM3, C and D show P450 nor. A and C show the secondary structure of the enzymes, with β-sheet shown in magenta, α-helices in cyan and loops in slate. The heme groups are also shown as red sticks. B and D show the enzymes in spacefill with red indicating negatively charged residues, blue: positive, green: hydrophilic and yellow: hydrophobic. Comparing the two enzymes shows the presence of the unusually large, positively charged region which in P450nor, which is notably absent in P450 BM3.

Figure 1.11

1.3.5.2 Peroxygenase systems

Another type of P450 redox system discovered in recent years utilises hydrogen peroxide as its electron source and, like P450nor, does not require additional redox partners. P450 SPα and P450 BSβ, from Sphingomonas paucimobilis and Bacillus subtilis, respectively, are capable of utilising hydrogen peroxide as a source of the electrons, protons and oxygen required for hydroxylation of their fatty-acid substrates (129-131). P450 SPα and BSβ are therefore designated as peroxygenase enzymes,
but sequence analysis has demonstrated that they belong to the P450 superfamily of enzymes (132). The structure of P450 BSβ has been solved and shows a typical P450 fold (133). However, a number of significant differences are also seen. For example, a positively charged arginine residue is found in the position typically occupied by a threonine in other P450 structures solved to date, but occupied by a histidine in the chloroperoxidase enzymes. The arginine is likely important for the splitting of the oxygen – oxygen bond of hydrogen peroxide (133). P450 SPα and BSβ are fatty acid hydroxylases, but uniquely in the P450s studied to date do not hydroxylate near or at the ω terminus of the fatty acid (129,130). Two of the most widely studied types of fatty acid hydroxylases, P450 BM3 and the family 4 P450s, hydroxylate around the ω terminus, with P450 BM3 typically oxygenating at ω-1 to ω-3 positions and the family 4 enzymes being ω-hydroxylases (134) (47). However, P450 SPα carries out 100% α-hydroxylation and P450 BSβ carries out a mixture of 60% β-hydroxylation with the other 40% α-hydroxylation (129,130). The structure of P450 BSβ also indicated the substrate-binding channel, which is stabilises substrate with a number of hydrophobic amino acids, and has an arginine located at the carboxylate end of the substrate forming stabilising electrostatic interactions (133). Structural and mutagenic studies of P450 BM3 have implicated R47 and Y51 in stabilising the carboxylate end of the fatty acid substrate at the top of the active site channel (76,135), instead P450 BSβ has a valine and leucine in these positions (133).

1.4 Catalytic cycle

A large amount of work has been carried out since the discovery of P450s in order to elucidate their catalytic mechanism. Electrons are usually delivered individually to the P450 via redox partners. The different redox systems employed are discussed in section 1.3 and essentially these systems ensure that 2 electrons are delivered to the cytochrome P450 individually at the relevant stages of the catalytic cycle. The catalytic cycle is summarised in Figure 1.12, with discussion of the intermediate states following.
Chapter 1 Introduction

Figure 1.12 Schematic representation of the cytochrome P450 catalytic cycle. The cycle progresses from the resting ferric low-spin water-bound form to the ferric high-spin form when substrate (RH) binds. Introduction of the first electron then becomes favourable, generating the ferrous substrate-bound form. Molecular oxygen then binds forming the dioxygen intermediate, which becomes the peroxo-iron form on further reduction. Introduction of a proton (probably from bulk solvent and delivered via one or more proximal amino acid side chains) generates the hydroperoxo-iron form. A second proton is then introduced, the molecular oxygen is split and water expelled, resulting in the iron-peroxo form, which rapidly collapses releasing hydroxylated product and returning the enzyme to the resting form (136,137).

1.4.1 Substrate-free enzyme

In its resting state the heme iron is in the ferric low-spin form, with a water molecule occupying the distal position on the heme iron (138,139), i.e. 4 of the 5 3d electrons are paired with one unpaired (S=½). The heme iron is therefore in a 6-coordinate form with the heme pyrrole nitrogens occupying the four equatorial positions. The thiolate ligand from the cysteine occupies the proximal axial position and the water occupies...
the distal axial position. These forms have been demonstrated both spectroscopically and structurally (116,140-142).

![Diagram](image)

**Figure 1.13**  *d-orbital electron configuration of low- and high-spin iron*

The splitting of the iron 3d-orbitals is shown in both the low- and high-spin forms. In the low-spin form four of the five 3d-electrons are paired giving a spin of \( \frac{1}{2} \), following binding of substrate the enzyme shifts to the high-spin form where all the 3d-electrons are unpaired \( i.e. \) \( S = \frac{5}{2} \).

### 1.4.2 Substrate binding

On substrate binding the distal water is displaced, the iron remains in the ferric form but shifts to high-spin, \( i.e. \) all 5 3d electrons become unpaired giving an \( S = \frac{5}{2} \) species. The spin shift can be monitored spectroscopically, with the low-spin species having a Soret band at \( \sim 418 \) nm and the high spin species having the Soret band at \( \sim 390 \) nm. Modi *et al.* have used NMR paramagnetic relaxation studies to study substrate binding to P450 BM3. They showed that substrate-binding is accompanied by an increase in the distance between the iron and the closest water molecule from 2.6 to 5.2 Å, consistent with dissociation of the distal water ligand from the heme iron (143). The spectral shift provides a useful spectroscopic tool and has been used extensively to calculate substrate dissociation constants (e.g.(144-146). Differences are also seen in the crystal structures of cytochrome P450 BM3 between substrate-free and substrate-bound forms. Comparisons of the crystal structure of P450 BM3 in the substrate-free form (142) with that of the palmitoleic acid-bound form (135) show large differences in the overall structure. There was a large motion of the substrate binding channel, moving from an open conformation to a closed one affecting a large number of the P450's amino acids in the region of the heme and suggesting potential roles for these residues in later stages of the catalytic cycle. A number of these amino acids have been
investigated by mutagenic studies and aspects of this work are discussed in section 1.4.2. Haines et al. have obtained a high resolution substrate-bound crystal structure of P450 BM3 and suggested that the water molecule which is distal to the heme iron in the substrate-bound form shifts away from the heme to another defined position in which it is bound by amino acid side chains, causing the changes seen between the substrate-bound and substrate-free crystal structures (147). It has however been shown that although the water molecule is probably important for the catalytic function of the enzyme, its displacement does not cause structural reorganisation (148). This aspect of the enzyme will be discussed in more detail in Chapter 3.

1.4.3 Introduction of first electron

The low- to high-spin switch has the effect of shifting the redox potential of the enzyme such that it is more feasible for an electron to be introduced into the system. In the low-spin form P450 cam has a redox potential of -303 mV (149) or -330 mV (150), but addition of substrate increases this to -173 mV (149) or -162 mV (150). In the case of P450 BM3 the redox potentials shift from -368 mV to -239 mV when substrate binds. NADPH, the electron donor for BM3 turnover, has a reduction potential of -320 mV (100), which is significantly more positive than the potential of substrate-free BM3 heme. This means it is thermodynamically unfavourable for the enzyme to turn over in this state, i.e. that electron transfer from NADPH through to the heme iron is disfavoured. The addition of substrate changes the redox potential such that the first electron can be delivered from NADPH through the redox partner, resulting in formation of a ferrous iron P450. This provides a control mechanism for the enzyme such that futile turnover of the enzyme (i.e. non-specific oxygen reduction) is inhibited in the absence of substrate, therefore preventing wastage of reducing equivalents.

1.4.4 The Oxyferrous complex

Following introduction of the first electron, oxygen can be bound to the heme iron. The ferrous oxy species is formed transiently, but a variety of techniques have been
used to study it. The binding of CO can be used as a model system for the study of the formation of the oxyferrous complex (151,152). It has been shown with the use of high pressure by Marchal et al. that CO binding presents a reliable model system for the study of oxygen binding in P450 BM3. Schlichting et al. solved the crystal structure of P450 cam in the dioxygen-bound intermediate form (137). This structure showed the dioxygen end on against the iron with an inter-atomic distance of 1.8 Å, and with the distal oxygen pointing towards T252, which is discussed in more detail in section 1.4.1. Ost et al. were able to generate a form of P450 BM3 which was stabilised in the oxyferrous form by mutating F393 to histidine or alanine (153,154). F393 is highly conserved in almost all cytochromes P450, and in the structure sits with its side chain against the cysteine responsible for the thiolate ligation to the heme iron (Cys 400 in P450 BM3) (142). Mutating this residue to histidine or alanine resulted in a BM3 variant which stabilised the oxyferrous complex by causing a positive shift of around 100 mV in both the substrate-free and substrate-bound heme iron redox potential relative to the wild-type enzyme (153,154). The stabilisation of the oxyferrous complex by mutation of just one amino acid demonstrates the fine balance in the catalytic cycle between the intermediate forms employed by the cytochromes P450. However, it should be noted that while the more positive heme iron reduction potential in the F393A/H mutants favoured NADPH-dependent reduction of the heme iron by the redox partner, enhanced stabilization of the ferrous-oxy complex results in lowered overall ability of the enzymes to catalyse fatty acid hydroxylation.
Figure 1.14  Heme, F393 and C400 of P450 BM3
The heme is shown in red sticks, with the iron represented in
spacefill. Below the plane of the heme C400 can be seen ligating to
the heme iron, F393 is seen to the left sitting 4.7 Å from C400.
Image generated using Weblab from PDB 1FAG (142).

1.4.5  Introduction of the second electron and first proton
Following introduction of molecular oxygen, a second electron is introduced into the
P450 heme iron. Davydov et al. have used low temperature electron paramagnetic
resonance (EPR) and electron nuclear double resonance (ENDOR) spectroscopies to
study species forming following the oxyferrous form in the catalytic cycle of P450
cam (136). They showed that reduction of the oxyferrous species yielded a peroxy-
iron form, with the iron in the ferric state. Following introduction of the second
electron the second proton may be introduced, yielding the hydroperoxo-iron species,
which has been shown to be a fundamental intermediate at the branch-point that leads
either to product formation or uncoupling of the cycle and futile loss of electrons to
oxygen (136). Of critical importance at this stage and subsequent stages of the
catalytic cycle is the thiolate proximal ligand to the heme iron (34).
1.4.6 Iron-peroxo species

The final species of the catalytic cycle, according to current studies, is the high-valence iron-oxo species, which attacks substrate and oxygenates it, rapidly releases product and returns the heme iron to its ferric resting form. The iron peroxo species forms when a second proton is taken up by the hydroperoxo iron and water is lost, splitting the molecular oxygen and leaving a single oxygen atom bound to the iron. This highly reactive species then rapidly oxygenates the proximally bound substrate (155). The iron-peroxo species has proven difficult to study, due to its short life, but a crystal structure of P450 cam putatively in this form is available, showing a single oxygen atom bound ~ 1.65 Å from the iron (137), although there is some disagreement as to whether this structure truly shows the iron-peroxo form (156). The leaving water molecule is also thought to be present in the structure obtained by Schlichting et al., resting against the I-helix, between residues T252 and G248 (137). It has also been suggested that T252 and D251 form a proton relay to pass the protons required during the catalytic cycle to the heme, and mutation of T252 to alanine resulted in significant loss of hydroxylation activity without concomitant reduction in the NADH and O₂ consumption, and therefore with an increase in the proportion of futile turnover by the enzyme (157). In the structure of the T252A mutant the A252 side chain moves away from the oxygen binding site and creates space for a new water molecule in the distal pocket. T252 is considered to play a role in stabilizing the oxy complex via hydrogen bonding with the oxygen and possibly also in protonation of the complex. In its absence protons may have to be sourced from elsewhere (possibly active site water molecules) and the less efficient proton delivery pathway probably allows for a greater proportion of the reactive oxy intermediates in the P450 cam catalytic cycle to collapse non-productively with production of damaging oxygen radicals or water. (158)
Chapter 1 Introduction

Figure 1.15 Structure of the putative iron-oxo intermediate of P450 cam.
The heme is shown in red, with the iron sitting in the centre. The oxygen (also red) can be seen in close proximity to the iron, above the heme plane. A water molecule sits to the right of the oxygen, and is thought to be the water lost in the previous stage of the catalytic cycle. Camphor is shown in purple. A number of amino acids implicated in this stage of the catalytic cycle are also shown, and labelled. Image created using Weblab from PDB 1DZ9 (137).

Studies of the thermophilic CYP119 have allowed closer examination of the iron-peroxo species. CYP119 is a thermostable P450 from Sulfolobus solfataricus and as such has a more rigid active site structure resulting in slower catalysis at room temperature (159). Using stopped-flow spectroscopy a species was seen with the same spectral properties as compound I (160), the ferryl-oxo porphyrin cation radical seen in a number of peroxidases, apparently confirming the presence of this species in the P450s (161,162).

The cysteine axial ligation has been shown to play a fundamental role in the splitting of molecular oxygen. It is in the deprotonated thiolate state, and protonation to the thiol form renders the enzyme inactive (11,35,163-165). Through investigation of sulphur distal ligands Sono et al. showed that the cysteine ligand to the heme iron creates a more electron-rich heme than seen in myoglobin, contributing to the large energetic “push” required for the splitting of molecular oxygen (166). Liu et al have shown the strong electron releasing character of the thiolate axial ligand with X-ray absorption near-edge studies of P450 cam, chloroperoxidase and myoglobin (167).
Ogliaro et al. have used theoretical techniques to show that removal of the thiolate ligation dramatically reduced the "push effect" of the thiolate ligation (168). Yoshioka et al. demonstrated that the basic amino acids Leu 358, Gly 359 and Gln 360 of P450 cam provide H-bonds to the proximal cysteinate, removal of which increases the $\sigma$-electron donation of the thiolate, decreasing the enzyme’s ability to split molecular oxygen (169).

**Figure 1.16** Axial heme iron thiolate ligation and stabilising amino acids of P450 cam

Shown in red is the heme group with the iron represented in spacefill. Cys357 can be seen ligating to the heme iron in the proximal position. The residues Leu 358, Gly 359 and Gln 360 are seen neighbouring Cys 357 and are thought to provide stabilising H-bonds. Image created using Weblab from PDB 2cpp (116).

### 1.4.7 Uncoupling

There are a number of routes by which the catalytic cycle can become uncoupled, resulting in wastage of electrons and no product formation. The principal uncoupling mechanism produces hydrogen peroxide, following the introduction of the second electron to the dioxygen form of the enzyme (157,170). It is also possible to utilise hydrogen peroxide to drive the P450 catalytic cycle, with direct conversion of the ferric heme iron to its hydroperoxo form, although this “peroxide shunt” mechanism is generally inefficient and oxidative destruction of the heme by the peroxide competes with the productive reaction. However, in recent studies Cirino and Arnold showed that P450 BM3 heme domain can catalyse the hydroxylation of fatty-acid using $\text{H}_2\text{O}_2$ as both a proton and electron source. However, the reaction occurred at severely
reduced rates and stability of the enzyme was impaired in the H$_2$O$_2$ environment by comparison with the physiological redox system (171). The catalytic cycle can also uncouple producing water or superoxide as is demonstrated in Figure 1.17 (170).

Figure 1.17 Possible routes of uncoupled P450 turnover. P450 catalytic cycle, as in Figure 1.12, but showing potential uncoupling reactions which result in the futile wastage of electrons (170). The uncoupling reactions are shown in green, with the productive catalytic cycle shown in blue.
P450 BM3 was first identified by Narhi and Fulco in 1982 (73). Fulco’s group had identified a fatty acid hydroxylase enzyme from *Bacillus megaterium* as early as 1974, but were unaware that the enzyme in question was a P450 (43). They identified that the enzyme is an NADPH- and oxygen-dependent system and found that supplementing additional redox partners did not increase activity, although removing endogenous ferredoxin-like activity resulted in the irreversible loss of P450 activity. At this stage Miura and Fulco also identified the enzyme as a ω-1, ω-2 and ω-3 fatty acid-hydroxylase (43). Since BM3 was identified as a P450 diflavin reductase fusion a great amount of research has been carried out on it, using it as a model system for the study of the redox system of eukaryotic endoplasmic reticulum P450s.

P450 BM3 is a soluble 119 kDa enzyme composed of two major domains, namely a 65 kDa reductase domain containing one FAD and one FMN, and a 54 kDa heme-containing P450 domain. BM3 is therefore a self-sufficient enzyme requiring only an electron source (NADPH) and oxygen to hydroxylate substrate (62).

P450 BM3 has been cloned in the intact and component domain forms and recombinantly expressed by a number of groups, allowing it to be extensively characterised (145,172,173).

P450 BM3 was found to be highly catalytically active, catalysing hydroxylation of the C20 unsaturated fatty acid arachidonate with a maximal rate of 17100 min⁻¹ and a $K_M$ of 4.7 μM, giving an overall catalytic efficiency of 3640 μM⁻¹ min⁻¹, this is the highest catalytic rate discovered for any monooxygenase P450 analysed to date (76).

The P450 BM3 crystal structure has been solved in both the substrate-free and substrate-bound forms. The solving of the structure of P450 BM3 heme domain with the mono-unsaturated fatty acid palmitoleic acid bound provided the first opportunity for comparison between the open and closed conformation of the substrate binding channel of a P450. Although the substrate-free and -bound structures of P450 cam (116,174) and substrate-bound structure of P450eryF (175) had previously been solved, these both showed the enzymes with their substrate access channels closed.
The structure of substrate-free BM3 heme domain was first solved in 1993 by Ravichandran et al. and showed an overall fold similar to P450 cam (and subsequently to other P450s), with a predominantly α-helical structure with a trigonal prism shape (142). As seen in the other P450 structures BM3 shows disruption in the I-helix between residues I263 and T269 with a water molecule preventing normal α-helical H-bonding between a number of these residues. Shown below in Figure 1.18 is the overall fold of P450 BM3 and following that in Figure 1.19 the overall fold of a number of other cytochromes P450.

Figure 1.18  **Overall secondary structural fold of substrate-free P450 BM3**  
Like all other P450s for which structural data is available P450 BM3 is a principally α-helical structure typical. The α-helices are shown as blue tubes, with β-sheets shown in cyan. Loops and random coil are shown in grey (PDB code: 2HPD (142)).
Chapter 1 Introduction

Figure 1.19 Secondary structure of CYP121, P450 cam, P450 Terp and P450 EryF

The secondary structure of each P450 is diagrammatically represented. CYP121 from Mycobacterium tuberculosis has been solved to high resolution, but its role remains unknown (PDB 1N40) (176). As has been discussed elsewhere P450 cam from Pseudomonas putida hydroxylates camphor and is the most studied P450 (PDB 2CPP) (116). P450 eryF from Saccaropolyspora erythraea participates in erythromycin biosynthesis (PDB 1OXA) (177). P450 terp is from another Pseudomonas species and hydroxylates terpineol as part of a pathway to utilise it as its sole carbon source (PDB 1CPT) (114,178). Each structure shows principally the same α-helical arrangement, resulting in a trigonal prism shape.
The structural data obtained by Li and Poulos, of palmitoleic acid-bound BM3 (135), when compared to the substrate-free structure as solved by Ravichandran et al., showed a number of important amino acids and movements carried out both for and as a result of substrate binding. A number of the findings have been studied by rational mutagenesis of residues hypothesised to be important; a number of these studies are discussed below.

Figure 1.20 Overlay of substrate-free and palmitoleate-bound BM3 structures
Overall fold of palmitoleate-bound BM3 heme domain (1FAG (135)), shown in green, and substrate-free BM3 heme domain (2HPD (142)) shown in blue. Comparison of the two structures shows changes in the overall fold of the enzymes, with the I-helix becoming more kinked on substrate binding, and large changes in the F and G-helices. The figure was generated using Weblab.

In substrate-bound structures there are a number of changes from the substrate-free structure. There is a large motion in the F and G helices, and the loop connecting them, leading to a conformational change in the H and I helices and the loops connecting the H and I helices, and the G to H helix. The FG loop forms one side of
the substrate access channel, with the other side formed by the β-sheet containing Arg 47. The motions in these two regions results in their moving together, causing the closing of the substrate access channel. This conformational change showed tyrosine 51 hydrogen bonding to the substrate carboxyl group and suggested that arginine 47 may also be doing so, although it was not well defined by the structure. Arginine 47 and tyrosine 51 have been mutated in a number of studies (76,179). Carmichael et al. showed that mutating R47 to leucine and Y51 to phenylalanine increased BM3's slow activity towards polycyclic aromatic hydrocarbons, but with greater uncoupling than seen with the wild-type enzyme, concluding that the residues are important for substrate docking (179). Noble et al. also mutated R47 and Y51, changing R47 to alanine and glycine and Y51 to phenylalanine. The R47G and R47A mutants showed decreased catalytic activity with fatty acid substrates, suggesting that electrostatic interactions between the guanidinium group of R47 and the fatty acid carboxyl group are essential for substrate binding. The Y51F mutant showed a higher $K_M$, but relatively unaffected $k_{cat}$. Noble et al. concluded that the tyrosine residue therefore played a role in substrate docking and possibly for transition-state stabilisation, but not at any other point in product formation (76). In the same study Noble et al. also investigated the role of phenylalanine 42, which appears to form a "cap" over the substrate channel after substrate has bound, protecting the substrate from the hydrophobic environment. Mutating F42 to alanine showed a vast increase in the $K_M$, but only a small decrease in the $k_{cat}$. They concluded that the absence of the phenylalanine was allowing solvent water more access to the active site and weakening the interactions between R47 and the substrate carboxyl group (76).
Figure 1.21 Position of F42, R47 and Y51 relative to the palmitoleic acid in molecule in BM3.
A number of other residues are shown which also contact the palmitoleic acid in the active site. The diagram was generated from the IFAG (135) substrate-bound structure and shows the carboxylate terminal of the palmitoleic acid in close proximity to F42, R47 and Y51, with the heme group seen below in red with its central iron atom represented in spacefill. Figure generated using Weblab.

The B' helix has been hypothesised to be important for substrate selectivity. It was predicted by Ravichandran et al. that phenylalanine 87 played an important role in substrate hydroxylation (142). This was confirmed in the palmitoleic acid-bound structure. Phenylalanine 87 was seen to have moved to a location where it blocks the ω-terminal methyl group of the heme from contacting the heme, causing it to “sit” in a hydrophobic pocket of residues including leucine 75, valine 78, isoleucine 263 and arginine 264, with its ω-methyl group contacting leucine 75 and valine 78.

Phenylalanine 87 has been mutated by various groups, yielding some contradictory results (171,180-182). In 1997 Oliver et al. mutated F87 to alanine, and using NMR paramagnetic relaxation measurements studied the effects of the mutation (181). The mutation had no significant effect on the catalytic efficiency of the enzyme or coupling, with laurate as substrate. They showed that the mutation altered the conformation of the substrate in the active site, with the substrate moving to 3.1 Å from the heme, as opposed to 5.1 Å in the wild type enzyme. They then tested the position of substrate hydroxylation and showed that the regiospecificity had shifted from a mix of ω-1, ω-2 and ω-3 to predominantly ω hydroxylation.
Chapter 1 Introduction

The same F87A mutation was also generated by Cirino and Arnold (171), but there results contradicted those found by Oliver et al. Cirino and Arnold were investigating the use of BM3 for biotechnological purposes and one problem for its industrial exploitation is the need for expensive NADPH by the enzyme. To address this they aimed to increase BM3's peroxxygenase activity, utilising the peroxide shunt to hydroxylate substrate. They mutated F87 to alanine to promote the enzyme's peroxxygenase activity and studied the effect of the mutation on the products formed. Using laurate as substrate with the intact enzyme, and NADPH as hydride donor they showed that the product formed by the enzyme was now principally ω-5, ω-4 and ω-3 hydroxylated, with some ω-2 and ω-1, but no detectable amounts of ω hydroxylation. They also found an increase in the amount of uncoupling in the enzyme and an increase in the peroxxygenase activity of the enzyme. They hypothesised that these changes may be due to the substrate being able to penetrate deeper into the enzyme's active site (171).

In a study of a number of active-site residues Noble et al. saw a significantly reduced rate of laurate-dependent NADPH oxidation by F87G BM3 using stopped-flow spectroscopy. They also saw that the system was less than 10% uncoupled, although this level was considerably greater than the wild-type enzyme (76).

Graham-Lorence et al. have also studied the role of F87 in arachidonic acid epoxidation, showing that replacement of the phenylalanine with valine resulted in greater regio- and stereo- specificity than that seen with the wild-type enzyme, but with more uncoupled turnover, concluding that F87 is responsible for gating of the heme bound oxidant (180).

Taken together the mutagenic studies on F87 have given some contradictory results. The study by Oliver et al. has been contradicted by a number of other studies, suggesting that their experiment, or data analysis, was in some way flawed. They did however monitor motion of the substrate, following binding and reduction, showing a motion to only 3.1 Å from the heme, around 2 Å closer than that in the wild-type enzyme (181). This suggests that the substrate is moving past the position which it occupies in the oxidised wild-type enzyme, consistent with the apparently altered
regio-selectivity of fatty acid oxidation in F87 mutants.

In further studies of the substrate-binding channel Ost et al. mutated a number of hydrophobic residues in the substrate access channel. Of particular interest was mutagenesis of L181, which appears to play an important role in substrate selectivity. Mutagenesis of L181 to lysine increased catalytic activity and decreased substrate-dissociation constants with shorter chain fatty acids, which BM3 normally only shows very limited activity towards (144). Sequence analysis showed that L181 is highly conserved in fatty-acid hydroxylating P450s, but is replaced by a phenylalanine in P450s which hydroxylate aromatic compounds as their substrate. Lindberg and Nagishi also studied the equivalent residues in the mouse cytochromes P45015α and P450co to which hydroxylate testosterone and coumarin respectively. P45015α and P450co only differ in 11 amino acids, yet have significantly different substrates. Mutagenesis of P209 in P450co to leucine, which is found in P45015α converted P450co from a coumarin hydroxylase to a testosterone hydroxylase enzyme (183). In addition to studying L181 Ost et al. also studied the residues L437, L75 and I236. They showed that mutagenesis of L437 to lysine or arginine reduced the enzymes ability to bind substrate and its catalytic activity, implicating this residue in substrate recognition. Mutation of L75 to threonine also had some effect on substrate specificity when in combination with the L181K mutation, but does not appear to play such a significant role as L181 or L437 in substrate recognition, mutagenesis of I263 also appeared to have very little effect on substrate specificity (144). The position of the residues L75, L181, I263 and L437 relative to the heme and palmitoleic acid in the structure resolved by Li and Poulos are shown below in Figure 1.22 (135).
Figure 1.22 Position of L75, L181, I263 and L437 relative to palmitoleic acid in P450 BM3

Palmitoleic acid can be seen in the centre of the figure with heme below in red, with the iron in spacefill representation. The hydrophobic residues L75, L181, I263 and L437 can be seen contacting to the palmitoleic acid, with F87, Y51 and R47 also shown. The figure was generated using Weblab from PDB 1FAG (135).

Alanine 264 and isoleucine 263 are found in the I-helix, where alanine 264 forms a hydrogen bond to threonine 268 causing a break in the normal helical hydrogen bonding (135,142), in the same manor as is seen in P450 cam (170). Arginine 264 is also hydrogen bonded to the distal water ligand. This means that arginine 264 is able to move 1Å away from the iron upon displacement of the water, which possibly results in the formation of the oxygen binding site (135). The crystal structure of camphor-bound P450 cam has been elucidated in the presence of carbon monoxide (184). There were certain problems accurately placing the CO molecule, but even with an error of up to 0.11 Å it is clear that the CO sits in the space created by the widening of the I-helix contacting T252 (corresponding to T268 in BM3), the side chain of which is moved slightly from the camphor-only structure to create a larger opening. The backbone of G248 (corresponding to A264 in BM3) H-bonds to T252 such that there is an opening in the I-helix, is also moved slightly, making a larger opening (184).
Figure 1.23  Camphor and CO bound P450 cam
The heme of P450 cam is shown in ball and stick representation with the heme iron in spacefill, CO can be seen binding directly to the heme iron. Camphor, T252 and G248 can also be seen, indicating their close proximity to the heme and CO molecule. The figure was generated using Weblab, from PDB 3CPP (184).

Threonine 268 has been mutated in BM3 to test this hypothesis by Yeom et al. (170). They generated the T268A mutant, which showed laurate-dependent NADPH oxidation was four times lower than wild type rates. Their study also showed 67% uncoupling, where reducing equivalents go to hydrogen peroxide production rather than substrate hydroxylation as described earlier. T268 does not appear to play any role in the stereo- or regio-specificity of the enzyme, since the same proportions of ω-1, ω-2 and ω-3 hydroxylated substrate were produced as wild-type BM3. The crystal structure of the enzyme showed no significant changes, except in the region of the mutation. Although there is no H-bond between A264 and T268A, as is seen in the wild type enzyme the carbonyl group of A264 is still strongly H-bonded to the distal water coordinated to the iron (170). Yeom and Sligar have also investigated the role of glutamate 267, by mutating the acidic glutamate to the amide residue glutamine. They showed that the rates of substrate hydroxylation were vastly decreased, and the proportion of ω-3 hydroxylated product significantly increased, especially using myristate as substrate. A new unidentified spectral species was seen during turnover, which has lead to the hypothesis that E267Q has created a new rate limiting step,
possibly with the glutamate contributing to the proton transfer pathway to the iron (185).

Figure 1.24  Palmitoleic acid-bound P450 BM3 showing the close proximity of residues F87, A264, T268 and G267 to the heme and palmitoleic acid

Shown as red sticks is the heme group with the central iron shown in spacefill representation, the palmitoleic acid can be seen above the heme with F87, A264m T268 and G267 in close proximity. Figure generated using Weblab (PDB: 1FAG (135))
1.6 **Aims and objectives**

This thesis describes the characterisation of a number of point mutations made to the model P450 system P450 BM3. The residue corresponding to A264 in P450 BM3 is highly conserved in all P450s, as either alanine or glycine, except in the family 4 P450s where a glutamate is found instead. It has been shown that this glutamate becomes covalently ligated to the 5'-methyl group of the heme. The initial aim of this work was to generate the equivalent A264E mutation in P450 BM3, which shows a high degree of homology to the family 4 P450s, with the hypothesis that this may yield a covalently ligated variant of P450 BM3. As is described in *Chapter 3* this mutation has been generated and characterised spectroscopically, kinetically and structurally, but instead of forming a covalently ligated heme has resulted in a novel Cys/Glu axial heme ligation state. Following generation of this novel heme ligation a series of other mutations have been made, with the aim of generating the novel or little studied Cys/His, Cys/Lys and Cys/Gln variants as described in *Chapter 4* and the Cys/Met and Cys/Cys variants as described in *Chapter 5*. *Chapter 6* describes the effects of pressure on these mutants, with the hypothesis that extremes of pressure may induce novel heme axial ligations.
Chapter 2  Methods
2.1 Molecular Biology

All molecular biology methods, where specific details are not given, were carried out by standard methods (186).

2.1.1 A264E Mutagenesis

The A264E mutant was constructed by overlapping mutagenic PCR (187). Three PCRs were carried out in total, using pBM20 as backbone in PCRs A and B. pBM20 contains the 1.4 kb gene encoding the heme domain of P450 BM3 in the expression vector pUC118 (145).

Oligonucleotides used for the generation of the A264E P450 BM3 mutant are shown in Table 2.1.

Table 2.1 Oligonucleotides used for creation of mutant A264E of P450 BM3.
The mutated base is shown in red and the restriction enzyme sites utilised for the mutagenesis (MfeI and BamHI – as indicated by primer names) are underlined.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MfeF</td>
<td>CGCTTGATAACATTGGTCTTTGC GG</td>
</tr>
<tr>
<td>BMutF</td>
<td>CTAAATTTGGGACACGAAACACAAACAGTGG</td>
</tr>
<tr>
<td>BMutR</td>
<td>CCACTTGGTTTTCGTGTCCTCCTCAGTAAG</td>
</tr>
<tr>
<td>BamR</td>
<td>AGGCAGACTCTAGAGATCCTATTTAGCG</td>
</tr>
</tbody>
</table>

Each PCR reaction for the generation of the A264E mutant was carried out in a 50 µl reaction volume with 20 pmol of each of the required oligonucleotides, 200 µM dNTPs, 1x PCR buffer and 200 ng backbone DNA. Finally, 1 unit of Clontech Advantage 2 DNA polymerase was added, which contains both a Taq DNA polymerase and a proofreading polymerase.

PCR A used the oligonucleotides MfeF, incorporating an MfeI restriction enzyme site
found 350 bp upstream of the mutation, and BmutR (the reverse transcript of the oligonucleotide BmutF) encompassing the mutagenic codon. The conditions used for PCR A are shown below in Table 2.2.

<table>
<thead>
<tr>
<th>Table 2.2</th>
<th>Conditions used for PCR A</th>
</tr>
</thead>
<tbody>
<tr>
<td>35 cycles of</td>
<td>95 °C 1 minute</td>
</tr>
<tr>
<td></td>
<td>52 °C 1 minute</td>
</tr>
<tr>
<td></td>
<td>72 °C 1 minute</td>
</tr>
<tr>
<td>1 cycle of</td>
<td>72 °C 3 minutes</td>
</tr>
</tbody>
</table>

The first oligonucleotide for PCR B, BMutF, was the forward transcript of oligonucleotide BmutR. The second oligonucleotide, BamR, incorporated a BamHI site 335 bp downstream of the mutation, see Table 2.1. The following conditions were used for the PCR (Table 2.3).

<table>
<thead>
<tr>
<th>Table 2.3</th>
<th>Conditions used for PCR B</th>
</tr>
</thead>
<tbody>
<tr>
<td>35 cycles of</td>
<td>95 °C 1 minute</td>
</tr>
<tr>
<td></td>
<td>52 °C 1 minute</td>
</tr>
<tr>
<td></td>
<td>72 °C 1.5 minutes</td>
</tr>
<tr>
<td>1 cycle of</td>
<td>72 °C 3 minutes</td>
</tr>
</tbody>
</table>

The products of PCRs A and B were run on a 0.8 % agarose gel and the correct band excised and purified from the gel using a QIAquick gel extraction kit (Qiagen) by the manufacturer’s protocol. PCRs A and B had therefore generated two DNA products, with PCR A generating a 350 bp DNA product encompassing the A264E mutation at the end of the sequence. PCR B generated a 335bp DNA product, with the mutation at the start of the sequence. PCR C combined the products of PCRs A and B, to generate a 988 bp DNA fragment from the MfeI restriction enzyme site upstream of the A264E mutation, to the BamHI site downstream of the mutation. Oligonucleotides MfeF and BamR (Table 2.1) were used with the following PCR conditions (Table 2.4).
Table 2.4 Conditions used for PCR C

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>95 °C</td>
<td>1 minute</td>
</tr>
<tr>
<td></td>
<td>52 °C</td>
<td>1 minute</td>
</tr>
<tr>
<td></td>
<td>72 °C</td>
<td>2 minutes</td>
</tr>
<tr>
<td>1</td>
<td>72 °C</td>
<td>3 minutes</td>
</tr>
</tbody>
</table>

Again the PCR products were run on a 0.8 % agarose gel stained with ethidium bromide. Once the correct band had been excised the product of PCR C was A-tailed and ligated into pGEM T according to the manufacturer’s instructions (Promega). This yielded plasmid pGEM-AE, which was transformed into JM109 ultra-competent cells (Stratagene) and screened for the inserted sequence by blue/white selection (186). 4 white colonies were selected, grown in 5 ml liquid culture containing 50 µg/ml ampicillin, miniprepped using a Qiagen miniprep kit, and verified by DNA sequencing by the dideoxy chain termination method at the PNACL facility (University of Leicester).

The selected pGEM-AE plasmid was digested with MscI and MfeI and the 520 bp band excised from an agarose gel and ligated into pBM20 and pBM23, which had also been digested with MscI and MfeI yielding 4.1 kb and 7.6 kb fragments respectively. The plasmid pBM23 contains the gene encoding flavocytochrome P450 BM3 and a 2 kb region encompassing the natural promoter from Bacillus megaterium, which is the naturally occurring segment immediately upstream of the BM3 gene (145). The ligation was optimised to use 100 ng backbone, 39 ng insert and ligated for 1 hour at 22°C with 0.3 units/µl T4 ligase (Promega). 39 ng insert was calculated to be 3 times the amount of backbone, i.e. three pieces of insert for each of backbone. The resultant plasmids were transformed into JM109 competent cells. The plasmid DNA was extracted from colonies using a Qiagen miniprep kit, digested to ensure it was contained the insert, and sequenced by the dideoxy chain termination method at the PNACL facility (University of Leicester).
### 2.1.2 A264K/H/QM/C mutagenesis

A264 mutants, excluding A264E, were created using the Stratagene QuikChange mutagenesis kit to mutate the heme domain of P450 BM3 in the plasmid pBM20, and intact flavocytochrome P450 BM3 in the plasmid pBM25. The plasmid pBM25 encodes the flavocytochrome P450 BM3 gene in the plasmid pUC118, but unlike the plasmid pBM23 does not contain the 2 kb \textit{B. megaterium} promoter-containing segment naturally found upstream of the flavocytochrome P450 BM3 gene. Primers used are detailed in Table 2.5. A BspHI restriction enzyme site was incorporated into the oligonucleotides used for the generation of the A264M, A264K and A264Q mutants by silent mutations, in order to verify the success of the PCR prior to sequencing. The oligonucleotides ultimately used for the generation of the A264C and A264H mutants did not contain the mutations required to introduce the BspHI restriction enzyme site. The primers were initially designed to include the silent mutations, but the PCR reactions were repeatedly unsuccessful, and so the oligonucleotides were redesigned without the silent mutations.

#### Table 2.5 Oligonucleotides used for the generation of A264K/M/Q/C and H mutations.

The BspHI restriction enzyme site introduced by mutagenesis is shown underlined, with silent mutations introduced to generate this site shown in blue. Mutations introduced to generate the relevant A264 amino acid changes are shown in red with the 264 codon highlighted.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer Name</th>
<th>Primer Sequence (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>A264K</td>
<td>A264KF</td>
<td>CATTCTTAATT\textcolor{red}{AAGGGT}CATGAAC\textcolor{blue}{ACAAAC}AAGTGG</td>
</tr>
<tr>
<td></td>
<td>A264KR</td>
<td>CC\textcolor{blue}{ACCTTTGTTTGTTCATGACCC}TTAATTAAGAATG</td>
</tr>
<tr>
<td>A264M</td>
<td>A264MF</td>
<td>CATTCTTAATT\textcolor{red}{ATGGGT}CATGAA\textcolor{blue}{ACAAAC}AAGTGG</td>
</tr>
<tr>
<td></td>
<td>A264MR</td>
<td>CC\textcolor{blue}{ACCTTTGTTTGTTCATGACCC}AATTAAGAATG</td>
</tr>
<tr>
<td>A264Q</td>
<td>A264QF</td>
<td>CATTCTTAATT\textcolor{red}{CAGGGT}CATGAA\textcolor{blue}{ACAAAC}AAGTGG</td>
</tr>
<tr>
<td></td>
<td>A264QR</td>
<td>CC\textcolor{blue}{ACCTTTGTTTGTTCATGACCC}TGATTAAGAATG</td>
</tr>
<tr>
<td>A264C</td>
<td>A264CF</td>
<td>CATTCTTAATT\textcolor{red}{TGTTGG}ACACG\textcolor{blue}{AACAAACA}AAGTGG</td>
</tr>
<tr>
<td></td>
<td>A264CR</td>
<td>CC\textcolor{blue}{ACCTTTGTTTGTTCATGACCC}AATTAAGAATG</td>
</tr>
<tr>
<td>A264H</td>
<td>A264HF</td>
<td>CATTCTTAATT\textcolor{red}{CATTGG}ACACG\textcolor{blue}{AACAAACA}AAGTGG</td>
</tr>
<tr>
<td></td>
<td>A264HR</td>
<td>CC\textcolor{blue}{ACCTTTGTTTGTTCATGACCC}AATTAAGAATG</td>
</tr>
</tbody>
</table>
PCR conditions were optimised and run as described in Table 2.6 to Table 2.10. Each PCR contained 5 μl of 10x reaction buffer, 50 ng pBM20 or pBM25, 125 ng of each oligonucleotide, 200 μM dNTPs and distilled water to 50 μl. Finally 2.5 U of Pfu turbo DNA polymerase were added and the PCR started.

Table 2.6  PCR conditions used for generation of the A264Q mutants in the heme domain and flavocytochrome P450 BM3

<table>
<thead>
<tr>
<th>A264Q PCR conditions</th>
<th>Heme domain</th>
<th>Intact flavocytochrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 cycle of 95 °C 30 s</td>
<td>1 cycle of 95 °C 1 min</td>
<td></td>
</tr>
<tr>
<td>16 cycles of 95 °C 30 s</td>
<td>4 cycles of 95 °C 30 s</td>
<td>45 °C 1 min 20 s</td>
</tr>
<tr>
<td>50 °C 1 min</td>
<td>68 °C 9 min</td>
<td></td>
</tr>
<tr>
<td>68 °C 4 min 40 s</td>
<td>14 cycles of 95 °C 30 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 °C 1 min 20 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td>68 °C 9 min</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.7  PCR condition used for the generation of the A264K mutants in the heme domain and flavocytochrome P450 BM3

<table>
<thead>
<tr>
<th>A264K PCR conditions</th>
<th>Heme domain</th>
<th>Intact flavocytochrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 cycle of 95 °C 30 s</td>
<td>1 cycle of 95 °C 30 s</td>
<td></td>
</tr>
<tr>
<td>12 cycles of 95 °C 30 s</td>
<td>18 cycles of 95 °C 30 s</td>
<td></td>
</tr>
<tr>
<td>55 °C 1 min</td>
<td>50 °C 1 min 10 s</td>
<td></td>
</tr>
<tr>
<td>68 °C 4 min 40 s</td>
<td>68 °C 9 min</td>
<td></td>
</tr>
</tbody>
</table>
**Table 2.8**  PCR conditions used for the generation of the A264M mutants in the heme domain and intact flavocytochrome P450 BM3

<table>
<thead>
<tr>
<th>A264M PCR conditions</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heme domain</strong></td>
<td><strong>Intact flavocytochrome</strong></td>
</tr>
<tr>
<td>1 cycle</td>
<td>1 cycle of</td>
</tr>
<tr>
<td>95 °C 30 s</td>
<td>95 °C 30 s</td>
</tr>
<tr>
<td>4 cycles</td>
<td>4 cycles of</td>
</tr>
<tr>
<td>95 °C 30 s</td>
<td>95 °C 30 s</td>
</tr>
<tr>
<td>45 °C 1 min 10 s</td>
<td>45 °C 1 min 10 s</td>
</tr>
<tr>
<td>68 °C 4 min 40 s</td>
<td>68 °C 9 min</td>
</tr>
<tr>
<td>12 cycles</td>
<td>16 cycles of</td>
</tr>
<tr>
<td>95 °C 30 s</td>
<td>95 °C 30 s</td>
</tr>
<tr>
<td>50 °C 1 min 10 s</td>
<td>50 °C 1 min 10 s</td>
</tr>
<tr>
<td>68 °C 4 min 40 s</td>
<td>68 °C 9 min</td>
</tr>
</tbody>
</table>

**Table 2.9**  PCR conditions used for the generation of the A264H mutants in the heme domain and intact flavocytochrome P450 BM3

<table>
<thead>
<tr>
<th>A264H PCR conditions</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heme domain</strong></td>
<td><strong>Intact flavocytochrome</strong></td>
</tr>
<tr>
<td>1 cycle of</td>
<td>1 cycle of</td>
</tr>
<tr>
<td>95 °C 30 s</td>
<td>95 °C 30 s</td>
</tr>
<tr>
<td>4 cycles of</td>
<td>4 cycles of</td>
</tr>
<tr>
<td>95 °C 30 s</td>
<td>95 °C 30 s</td>
</tr>
<tr>
<td>45 °C 1 min 20 s</td>
<td>45 °C 1 min 20 s</td>
</tr>
<tr>
<td>68 °C 5 min 40 s</td>
<td>68 °C 9 min</td>
</tr>
<tr>
<td>12 cycles of</td>
<td>16 cycles of</td>
</tr>
<tr>
<td>95 °C 30 s</td>
<td>95 °C 30 s</td>
</tr>
<tr>
<td>50 °C 1 min 20 s</td>
<td>50 °C 1 min 20 s</td>
</tr>
<tr>
<td>68 °C 5 min 40 s</td>
<td>68 °C 9 min</td>
</tr>
</tbody>
</table>
Chapter 2 Methods

Table 2.10 PCR conditions used for the generation of the A264C mutants in the heme domain and intact flavocytochrome P450 BM3

<table>
<thead>
<tr>
<th>A264C PCR conditions</th>
<th>Intact flavocytochrome</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heme domain</strong></td>
<td><strong>Intact flavocytochrome</strong></td>
</tr>
<tr>
<td>1 cycle of 95 °C 30 s</td>
<td>1 cycle of 95 °C 30 s</td>
</tr>
<tr>
<td>16 cycles of 95 °C 30 s</td>
<td>4 cycles of 95 °C 30 s</td>
</tr>
<tr>
<td>50 °C 1 min 20 s</td>
<td>45 °C 1 min 20 s</td>
</tr>
<tr>
<td>68 °C 5 min 40 s</td>
<td>68 °C 9 min</td>
</tr>
<tr>
<td>14 cycles of 95 °C 30 s</td>
<td>50 °C 1 min 20 s</td>
</tr>
<tr>
<td></td>
<td>68 °C 9 min</td>
</tr>
</tbody>
</table>

5μl of each PCR product was run on a 0.8 % agarose gel against NEB 1kb DNA ladder. Where DNA was present at ~ 4.1 kb or ~ 6 kb, for mutagenesis of the pBM20 or pBM25 plasmids respectively, the remaining PCR product was digested for at least 1 hour at 37 °C with the restriction enzyme DpnI. DpnI digests only methylated DNA. pBM20 and pBM25 used for mutagenesis were isolated following transformation into TG1 cells, which contains the Dam methylase gene. The DNA is therefore methylated. DNA generated in the PCR reaction is not methylated and therefore not digested by DpnI. The PCR product was then transformed into JM109 competent cells (Stratagene) and six colonies miniprepped using a Qiagen miniprep kit. The concentration of the plasmid DNA was estimated by 0.8 % agarose gel electrophoresis. The A264K, A264M and A264Q mutants were then digested with the restriction enzyme BspHI, exploiting a restriction site that had been introduced into the mutagenic primers. The presence of an extra BspHI restriction enzyme site, yielding the correct sized DNA fragments, verified the presence of the mutation. DNA was then sequenced by the dideoxy chain termination method at the PNACL facility (University of Leicester). Primers used for DNA sequencing are detailed below in Table 2.11.
Table 2.11 Primers used for DNA sequencing

<table>
<thead>
<tr>
<th></th>
<th>5'</th>
<th>3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heme domain</td>
<td>TTC ACA</td>
<td>CAG GAA</td>
</tr>
<tr>
<td></td>
<td>ACA GCT</td>
<td>AT</td>
</tr>
<tr>
<td></td>
<td>TCC TGC</td>
<td>GTT TTC</td>
</tr>
<tr>
<td></td>
<td>CCT ATA</td>
<td>T</td>
</tr>
<tr>
<td></td>
<td>TAC CGG</td>
<td>AAG ACA</td>
</tr>
<tr>
<td></td>
<td>TGA CAC</td>
<td>G</td>
</tr>
<tr>
<td>FMN domain</td>
<td>TGG CGG</td>
<td>TAT TCC</td>
</tr>
<tr>
<td></td>
<td>TCC TTC</td>
<td>ACC TA</td>
</tr>
<tr>
<td></td>
<td>ATG GCG</td>
<td>TGA ACA</td>
</tr>
<tr>
<td></td>
<td>TAT GTG</td>
<td>GA</td>
</tr>
<tr>
<td>FAD domain</td>
<td>TGG CTG</td>
<td>CTA AAA</td>
</tr>
<tr>
<td></td>
<td>CGG TCT</td>
<td>GCC</td>
</tr>
<tr>
<td></td>
<td>CTT CGG</td>
<td>CTG CCG</td>
</tr>
<tr>
<td></td>
<td>TTC ACC</td>
<td>T</td>
</tr>
</tbody>
</table>

### 2.2 Enzyme over-expression and purification

#### 2.2.1 Overexpression

Wild type P450 BM3, and mutants in both heme domain and intact forms, were overexpressed by the same protocol. Plasmid was transformed into actively growing TG1 cells and single colonies grown to an OD$_{600}$ of 0.5 in LB medium before being frozen in 20% glycerol at -80°C. 100 ml overnight cultures were inoculated from the glycerol stocks and grown in LB medium containing 50 μg/ml carbenicillin. 2 l Erlenmeyer flasks containing 500 ml Terrific broth and 75 μg/ml ampicillin were inoculated with 5ml of the starter culture. The culture was then grown for 36 hours at 37 °C with shaking at 250 rpm.

#### 2.2.2 Cell Harvesting and Lysis

Cells were harvested by centrifugation at 6,000 rpm, the supernatant was discarded and the cell pellets were washed by resuspension in ice cold 50 mM Tris HCl, 1 mM EDTA buffer at pH 7.2 (buffer A) before re-centrifugation as previously. Pelleted cells were then frozen at -20 °C. Cell lysis was carried out first by 2 passes through a French press at 950 psi followed by sonnication in 20 second bursts, with a break.
between bursts of at least 2 minutes to allow cooling of cell lysate. The cell lysate was kept ice cold at all times and 1 mM benzamidine was added prior to beginning lysis.

Cellular debris was removed by centrifugation (18,000 rpm, 20 minutes, 4 °C) and the supernatant was dialysed twice against 10 l (2 x 5 l) buffer A containing benzamidine (1 mM) for at least 4 hours each time.

2.2.3 Purification

Heme domain, in both wild-type and mutant forms, was purified in a 3 step process with the anion exchange columns DEAE-Sepharose (diethylaminoethyl-Sepharose), hydroxyapatite and Q-Sepharose (quaternary amino-Sepharose). Intact flavocytochrome enzymes were purified in a 2 step process using DEAE-Sepharose followed by the NAD(P)H affinity column mimic yellow.

2.2.3.1 DEAE-Sepharose

A DEAE anion exchange column, which had been pre-equilibrated in buffer A was used for initial purification of both heme domain and intact flavocytochrome, in wild-type and mutant forms. DEAE Sepharose resin was purchased from Amersham-Pharmacia and poured to generate a 15 cm height column, as per the manufacturer's instructions, in a column with internal diameter of 26 mm, also purchased from Amersham-Pharmacia. Following loading, the protein was washed with at least 2 column volumes of buffer A before eluting the protein with a linear salt gradient from 0 to 500 mM KCl in buffer A. Fractions were analysed spectroscopically by comparison of the total protein content (A$_{280}$) and the P450 Soret band (A$_{418}$). Those selected on the basis of highest A$_{418}$/A$_{280}$ ratio were concentrated by ultrafiltration with centriprep YM30s (Millipore) prior to being dialysed extensively into 25 mM potassium phosphate buffer at pH 6.5 (Buffer B) at 4 °C.
2.2.3.2 Hydroxyapatite

Ceramic hydroxyapatite column resin, purchased from Biorad, was prepared as per the manufacturer’s instructions and poured to generate a ~ 10 cm column with an internal diameter of 26 mm, purchased from Amersham-Pharmacia. Following initial purification by DEAE anion exchange chromatography, heme domain mutants and wild-type P450 BM3 proteins were loaded onto a hydroxyapatite anion exchange column, with both protein and column pre-equilibrated in buffer B. After washing the column in several volumes of buffer B the protein was eluted with a linear gradient of buffer B against 500 mM KPi pH 6.5. The fractions were then selected, pooled and concentrated as previously, before dialysing into buffer A again.

2.2.3.3 Q-Sepharose

Q-Sepharose resin was purchased from Amersham-Pharmacia and prepared in the same way as DEAE-Sepharose. For purification of wild type heme domain and mutant P450 BM3 enzymes, protein was then loaded onto a third anion exchange column, this time a Q-Sepharose column, and further purified using the same protocol as that used with the DEAE column. As previously, column peak fractions were selected and pooled together. Once fully purified the protein was extensively dialysed against buffer A and concentrated to >300 μM, before one final dialysis against buffer A containing 50% glycerol, prior to storage at -80°C.

2.2.3.4 Mimetic Yellow

The mimetic yellow resin (ProMetic Biosciences) was poured to generate a 10 cm column with internal diameter of 16 mm (column purchased from Sigma-Aldrich). Wild-type and mutant flavocytochrome BM3 enzymes were dialysed into buffer B and loaded on a mimetic yellow affinity column pre-equilibrated in buffer B, as a second purification step following DEAE-Sepharose. The column was then washed extensively until all unbound protein was removed, verified spectroscopically by following A280 of the eluent. The protein was eluted in a single step with 500 mM NaCl containing 25 mM 2’ adenosine monophospate (2’ AMP). The product was concentrated by ultrafiltration to >300 μM and dialysed against sufficient quantities of
buffer A to remove all NaCl and 2' AMP. The protein was then dialysed against 50% (v/v) glycerol in buffer A and stored at -80°C.

### 2.2.3.5 Protein for X-ray Crystallography use

Instead of dialysing into 50% glycerol in buffer A at the final stage of protein isolation, samples for use in crystallography were dialysed against 10 mM Tris HCl, pH 7.4 and used immediately to set up crystal trials.

### 2.3 SDS-PAGE

The heme domain of P450 BM3 was analysed for purity using 10% SDS-PAGE and intact flavocytochrome BM3 using 6% SDS-PAGE, both by the methods described in Sambrook et al. (186). Samples were run alongside pre-stained broad range marker (NEB), and electrophoresis gels were stained with coomassie brilliant blue stain by the method of Sambrook et al. (186). To assess if any covalent heme ligation had formed, SDS-PAGE gels were stained for the presence of heme by the method of Thomas et al. (188). Gels were immersed in 0.45 mg/ml TMBZ (3,3',5,5',-tetramethylbenzidine dissolved in methanol) and 100 mM sodium acetate buffer at pH 5. The gels were incubated in the dark in this solution for 1 hour, before hydrogen peroxide was added to a final concentration of 30 mM. Over the next 5 minutes bands developed in the gel. When the bands reached suitable levels of intensity, the reaction was halted with the addition of 30 ml isopropanol. The gels were photographed prior to staining with coomassie brilliant blue and photographing again.

### 2.4 Protein concentration calculations

#### 2.4.1 Reduced/CO adduct formation

All UV-visible spectroscopy was carried out on a Varian Cary 50 UV-visible spectrophotometer using a quartz cuvette with a path length of 1 cm, unless otherwise stated.
The concentration of proteins was calculated spectroscopically using the Beer Lambert law by the method of Omura and Sato (189).

\[ A = \varepsilon cl \]

**Equation 1** The Beer-Lambert law for the calculation of the concentration of light absorbing compounds
Where \( A \) is the observed absorbance, \( \varepsilon \) the extinction coefficient for the sample, \( c \) the sample concentration, and \( l \) the sample pathlength.

Enzyme was reduced with sodium dithionite, giving the ferrous form, before being gently bubbled with carbon monoxide. Difference spectra of ferrous/CO bound – ferrous protein were then constructed by subtracting the spectrum of the reduced enzyme from that of the CO complex, and the absorbance difference calculated between 450 nm and 490 nm. The extinction coefficient \( \varepsilon_{490-450} = 91 \text{ mM}^{-1}\text{cm}^{-1} \) was used to determine the P450 concentration.

### 2.4.2 Pyridine hemochromagen

Heme protein concentration was also calculated using the method of Berry and Trumpower (190). Spectra were collected for a P450 sample containing 100 mM NaOH, 20 % (v/v) pyridine and 300 \( \mu \)M \( \text{K}_3\text{Fe(CN)}_6 \) in a sealed cuvette. The sample was then reduced by the addition of a few grains of sodium dithionite, the cuvette re-sealed and a spectrum collected. The heme concentration was then calculated using the Beer-Lambert law, as shown in Equation 1, with the extinction coefficient of \( \varepsilon_{550-535} = 24 \text{ mM}^{-1}\text{cm}^{-1} \).

### 2.5 Substrate and inhibitor binding studies

The substrates arachidonic acid, palmitoleic acid, palmitic acid, myristic acid and lauric acid, and the inhibitors 4-phenyl imidazole and sodium cyanide were titrated against approximately 5 \( \mu \)M wild-type and/or mutant P450 BM3 heme domain to determine \( K_d \) values from spectral shifts. All binding titrations were carried out at 30
°C using 25 mM MOPS buffer with 100 mM KCl at pH 7.4 (assay buffer), with enzyme concentration less than 5 µM and in a total volume of 1 ml. Substrate was added as 0.1 µl aliquots from a stock solution as described in Table 2.12, until no further spectral change was observed. Difference spectra were then generated by subtraction of the original spectrum from subsequent spectra and the maximal absorption difference (using identical wavelength pairs in each case at peak and trough of the difference spectra) plotted against the corresponding ligand concentration. The data points were then fitted to a rectangular hyperbola, allowing calculation of a binding constant. In certain cases, data were fitted to a quadratic equation, when substrate binding gave a $K_d$ less than 5 times the enzyme concentration. The equations for a rectangular hyperbola is shown in Equation 2 and the tight binding quadratic equation in Equation 3. The quadratic function provides a more accurate estimate of the $K_d$ value for very tight binding inhibitors/substrates. Typically, the values predicted using the two different functions converge at $K_d$ values ~ 5 x the enzyme concentration used.

$$\text{Abs} = \frac{A_{\text{max}} [S]}{K_d + [S]}$$

Equation 2 Rectangular hyperbola equation used to calculate the $K_d$ for substrate or inhibitor binding to P450 BM3 enzymes

$A_{\text{max}}$ is the maximal absorbance change, $K_d$ the dissociation constant and Abs is the observed absorption difference observed at the substrate concentration $[S]$.

$$A_{\text{obs}} = \left( \frac{A_{\text{max}}}{2Et} \right) \star \left( \left( S + Et + K_d \right)^2 - \left( S + Et + K_d \right) - (4 \star Et \star X)^{0.5} \right)$$

Equation 3 Quadratic equation

The quadratic equation is used in place of the rectangular hyperbola where the apparent dissociation constant is less than 5 times the enzyme concentration. $A_{\text{max}}$ is the maximal absorbance, $K_d$ the apparent dissociation constant, $Et$ the enzyme concentration used and $S$ the substrate concentration at which the absorption reading $A_{\text{obs}}$ is made.
Table 2.12  Substrate and inhibitor stock concentrations and the solvents used

<table>
<thead>
<tr>
<th>Substrate/Inhibitor</th>
<th>Solvent</th>
<th>Stock concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonic acid</td>
<td>Ethanol</td>
<td>33 mM</td>
</tr>
<tr>
<td>Palmitoleic acid</td>
<td>Ethanol</td>
<td>19.2 mM</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>Acidified ethanol</td>
<td>20 mM</td>
</tr>
<tr>
<td>Myristic acid</td>
<td>Acidified ethanol</td>
<td>30 mM</td>
</tr>
<tr>
<td>Lauric acid</td>
<td>Acidified ethanol</td>
<td>50 mM</td>
</tr>
<tr>
<td>4-Phenylimidazole</td>
<td>Assay buffer</td>
<td>1 mM</td>
</tr>
<tr>
<td>Sodium cyanide</td>
<td>Assay buffer</td>
<td>1-10 M</td>
</tr>
</tbody>
</table>

As can be see in Table 2.12 acidified ethanol was used as solvent for a number of substrates, the acid HC1 was maintained at less than 1 % of the stock substrate solution.

2.6  Steady state kinetics

Steady state kinetic studies were carried out with the flavocytochrome form of wild-type and mutant P450 BM3, using the same substrates as those used for the binding titrations.

Assays were carried out in assay buffer (20 mM MOPS, 100 mM KCl, pH 7.4). Typically 10 – 100 nmol of enzyme were used per assay, with NADPH at near-saturating concentrations (200 μM). Enzyme and substrate were mixed in assay buffer and allowed to equilibrate to 30 °C, the reaction was then initiated by addition of NADPH and mixing, and the absorbance change at 340 nm monitored. Measurements were repeated at each substrate concentration at least 3 times. The rate of NADPH oxidation was calculated using the extinction coefficient of 6210 M⁻¹cm⁻¹. Plotting the initial rate from each kinetic measurement against substrate concentration gave a curve from which the $k_{cat}$ and $K_M$ can be calculated, using the Michaelis Menten equation.
\[ V = \frac{V_{\text{max}}[S]}{K_M + [S]} \]

Equation 4  Michaelis-Menten equation  
\( K_M \) is the Michaelis constant, describing substrate concentration at the half maximal rate of the reaction. \( V_{\text{max}} \) is the maximal rate of the reaction and \( S \) the substrate concentration.

2.7 Hydrogen peroxide and superoxide measurement

The amount of uncoupled turnover was measured essentially as described by Macheroux et al. (191) and Noble et al. (76). 0.15 to 0.2 \( \mu \)M mutant and wild-type intact flavocytochrome P450 BM3 enzymes were incubated both with and without 950 \( \mu \)M laurate in assay buffer with 200 \( \mu \)M NADPH. The oxidation of NADPH was monitored at 340 nm and once completely consumed 400 \( \mu \)M \( o \)-dianisidine was added (from an 8 mM stock prepared in 20 % (v/v) Triton X 100) along with 0.1 \( \mu \)M horseradish peroxidase. Horseradish peroxidase catalyses the breakdown of hydrogen peroxide to oxygen and water, oxidising \( o \)-dianisidine as its substrate. In the oxidised form \( o \)-dianisidine has an absorbance at 440 nm. Using the absorption coefficient of \( \Delta \varepsilon_{440} = 11600 \text{ M}^{-1} \text{ cm}^{-1} \) the concentration of hydrogen peroxide produced by P450 BM3 can be calculated. To ascertain the amount of uncoupled turnover generating superoxide, 0.1 \( \mu \)M superoxide dismutase was also added prior to the addition of NADPH, converting the superoxide to hydrogen peroxide. Subtracting the quantity of hydrogen peroxide produced without the addition of SOD allowed calculation of the amount due to superoxide formation and conversion to peroxide by SOD.

2.8 Redox potentiometry

All redox titrations were carried out in a Belle technology glove box under a nitrogen atmosphere, with a UV-visible probe inside the box attached to a Varian Cary 50 UV-visible spectrophotometer. Potentials were measured with a Thermo Russell electrode attached to a Hanna pH211 microprocessor meter. Redox titrations were carried out for
both wild type heme domain of P450 BM3 and for mutant heme domains. Titrations were carried out using the arachidonate-bound and substrate-free forms of the enzyme essentially by the method of Dutton (192). All redox titrations were carried out in 100 mM KPi at pH 7.0 containing 10 % (v/v) glycerol (redox buffer), which had been deoxygenated by bubbling extensively with O2-free argon. Traces of oxygen were removed from the protein samples by passing them through a Bio-Rad Econo-pac 10DG gel filtration column within the glove box, which had been pre-equilibrated with redox buffer. All redox potentials were carried out in 5 ml volume with 5-10 μM enzyme concentration. Mediators were added to the enzyme solution to speed equilibration between the enzyme and electrode, 1.5 μM methyl viologen, 2.5 μM benzyl viologen, 7 μM 2-hydroxy-1,4-naphthaquinone and 5 μM phenazine methosulfate. The reductant sodium dithionite, in a ~ 10 mM stock, was titrated against the enzyme solution by addition of very small volumes of the reductant, and the enzyme sample was left to equilibrate, typically for 10 – 15 minutes, prior to each spectrum being collected and the potential being noted at each titration point. When the enzyme was entirely reduced, potassium ferricyanide was added to reoxidise it to the start point and a final reading was taken. 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 Nernst function was then fitted to the data and the midpoint potential calculated from it. The one electron Nernst equation is shown in Equation 5. In substrate-bound titrations, arachidonate was added as aliquots from a 33 mM stock in ethanol until no more high-spin enzyme was formed (as observed by lack of further optical change), prior to the reductive titration being started.
Equation 5  
\[ A = \frac{A_{\text{Abs}} + B_{\text{Abs}} \cdot 10^{\left(\frac{(E_o - X)}{C}\right)}}{1 + 10^{\left(\frac{(E_o - X)}{C}\right)}} \]

**One electron Nernst equation**

\( A_{\text{Abs}} \) is the absorbance for the ferric P450 form and \( B_{\text{Abs}} \) the absorbance of the ferrous P450, \( E_o \) is the reduction potential for the \( \text{Fe}^{3+}/\text{Fe}^{2+} \) transition and \( X \) the applied potential. \( C \) is a constant (59 mV for a one electron reduction at 298 K) derived from the gas constant \( R = 8.315 \text{ J mol}^{-1} \text{ K}^{-1} \), the absolute temperature (in K) and Faraday's constant \( (96500 \text{ C mol}^{-1}) \) representing the term \( RT/nF \), where \( n \) is the number of electrons.

### 2.9  
**Denaturation studies**

#### 2.9.1 Temperature effects on wild-type and mutant P450 BM3 heme domain spectral properties

The effect of changes in temperature were studied in the A264E mutant and wild-type heme domains. UV-visible spectra were collected from 250 – 700 nm for 4 μM enzyme in assay buffer at 2°C temperature intervals from 6°C to 60°C, with 2 minute incubations at each temperature prior to spectral acquisition.

#### 2.9.2 pH effects on wild-type and mutant BM3 heme domain spectral properties

The effect of pH on mutant and wild-type heme domains was studied by UV-visible spectroscopy between wavelengths of 250-700 nm. Spectra were collected for 4 μM enzyme in 100 mM KPi buffer at pH 5 to 9, with intervals of 1 pH unit. As above, 2 minutes equilibration was allowed prior to recording spectra at each pH value.

#### 2.9.3 Ionic strength effects on wild-type and mutant BM3 heme domain spectral properties

Ionic strength effects on the UV-visible spectral properties of wild-type and mutant heme domain enzymes were studied. Samples were prepared in 20 mM MOPS at pH
Chapter 2 Methods

7.4, with enzyme concentration between 3 and 5 μM. KCl was added from a 1 M stock, also in 20 mM MOPS at pH 7.4, to concentrations between 0 and 1 M KCl, at 0.1 M intervals. As above, 2 minutes equilibration was allowed prior to recording spectra at each ionic strength.

2.9.4 Guanidine denaturation of A264E heme domain

The denaturing effects of guanidine hydrochloride (GdnHCl) were studied on the heme domain of A264E BM3 by UV-visible and fluorescence spectroscopy. 3 μM enzyme was incubated in assay buffer for 10 minutes with GdnHCl added at concentrations between 0 and 6 M at 0.5 M intervals. Tryptophan fluorescence was measured with an excitation wavelength of 280 nm and emission collected between 300 and 400 nm, using a Varian Cary Eclipse fluorescence spectrophotometer. UV-visible spectra were collected between 250 and 700 nm for the same samples.

The P450/P420 spectral equilibrium and its dependence on GdnHCl concentration was also studied by UV-visible spectroscopy. Samples were incubated with GdnHCl as described above, before excess dithionite was added and the sample was introduced into a carbon monoxide environment, allowing CO to bind. UV-visible spectra were then collected for the samples as described above.

2.10 Circular dichroism

Circular dichroism spectra were collected in both the visible, near UV and far UV regions for the A264E mutant using a Jasco J715 specropolarimeter. Far UV spectra were collected from 190–260 nm using a cylindrical cell of pathlength 0.01 cm, holding 300 μl of sample. The spectra were collected with a scanning rate of 10 nm min⁻¹ and 5 spectra averaged for each sample. Spectra were collected for oxidised, arachidonate-bound and 4-phenylimidazole-bound enzyme using 1.8 μM enzyme. Substrate binding was verified by collection of a UV-visible spectrum prior to collection of the CD spectrum.
Chapter 2 Methods

A 3 ml cell with a 1 cm pathlength was used to collect visible-near UV CD spectra from 260 to 600 nm. Spectra were recorded at a scan rate of 20 nm min\(^{-1}\) and 5 spectra were accumulated and averaged, for each sample. 45 μM enzyme was used with each sample. As before substrate-free, arachidonate-bound and 4-phenyl imidazole-bound spectra were collected.

2.11 Electron Paramagnetic Resonance (EPR)

Wild-type and A264E heme domain electron paramagnetic resonance (EPR) spectra were collected in collaboration with Dr Myles Cheesman (Dept of Chemistry, University of East Anglia). All other mutant P450 EPR spectra were collected by Dr Harriet Seward (Dept of Biochemistry, University of Leicester) using facilities in the Department of Chemistry, University of East Anglia. Spectra were generated on a Bruker EPR spectrometer at a temperature of 10 K, and at a frequency of approximately 10 GHz with a power of 2 mW. All enzyme samples were between 500 μM and 1 mM concentration in pH 7.4, 50 mM Tris HCl containing 1mM EDTA and 50% glycerol (v/v). Samples were loaded into EPR tubes and frozen in liquid nitrogen before being placed in the spectrometer and spectra collected. Spectra were collected in the absence of substrate and following the addition of substrate and inhibitors as detailed in the relevant Results chapter. Derivative spectra were automatically generated and g-values determined from them.

2.12 Magnetic circular dichroism (MCD)

Magnetic circular dichroism of the A264E mutant was carried out by Dr Myles Cheesman (Department of Chemistry, University of East Anglia) and by Dr Harriet Seward (Department of Biochemistry, University of Leicester) using facilities at the Department of Chemistry, University of East Anglia. Spectra were recorded for the wild-type and mutant P450 BM3 heme domains on Jasco circular dichrograph models J-810 and J-730 for the UV-visible and near-infrared regions, respectively. UV-visible spectra were recorded in a 0.1 cm pathlength quartz cuvette with sample concentrations between 50 and 200 μM. Near-infrared spectra were recorded in a 0.2
cm pathlength quartz cuvette with sample at the same concentrations as those used to collect EPR spectra. An Oxford Instruments superconducting solenoid with a 25 mm ambient bore was used to generate a magnetic field of 6 Tesla. To record MCD through the 1400–2000 nm region, background absorption caused by vibrational overtones was minimized by preparing samples in deuterium oxide solutions. Samples were prepared in 20 mM HEPES made up in deuterium oxide (pH 7.5).

2.13 Resonance Raman

Resonance Raman spectra were collected in collaboration with Dr Rachael Littleford or Dr John Clarkson (Dept of Pure and Applied Chemistry, University of Strathclyde, Glasgow). Spectra were collected for heme domains of wild-type and mutant P450 BM3 enzymes at a concentration of 50 μM in the substrate-free and arachidonate-bound forms and at ambient temperature. A 15 mW 406.7 nm radiation source was used at the sample from a Coherent Innova 300 Krypton ion laser, with spectra acquired with a Renishaw micro-Raman system 100 spectrometer. The samples were held in a capillary under the microscope with 5 x 15 seconds exposure.

2.14 Product and substrate analysis

Product formation was measured using mass spectrometry. 0.4 μM wild type and mutant flavocytochrome enzymes were incubated for 14 hours at room temperature with 45 μM lauric acid and 600 μM NAPDH, with stirring in assay buffer. Negative controls were also prepared identically to the above reactions, but without the addition of NADPH. The reaction was halted by acidification to pH 2 with 1 M hydrochloric acid. Fatty acids were extracted from the aqueous environment into one volume of dichloromethane. The lower layer, which contained the dichloromethane and fatty acids, was extracted and remaining aqueous material removed by the addition of solid magnesium sulphate. The solution was then filtered and the dichloromethane evaporated, leaving the fatty acid dried to the tube. The fatty acids were then resuspended in ~ 1 ml methanol. 20 μl samples were injected into a E1 (70 eV ionisation) Micromass Quatro triple quadrupole mass spectrometer, a common access
facility in the Department of Chemistry, University of Leicester. Samples from the aqueous layer were also run to ensure complete extraction had been achieved.

2.15 Crystallisation of A264E heme domain

Crystals were grown of A264E heme domain, using the sitting drop technique at 5°C in Linbro boxes. An initial screen of a number of precipitants and salts was used with the buffer cacodylic acid at pH 6.3. 2 μl of mother liquor was added to 2 μl of a 15 mg/ml protein solution in 10 mM Tris HCl buffer at pH 7.4. Trays were incubated at 5 °C and monitored daily for crystal growth. Following the initial screen, trays were set up using the salt manganese sulfate at concentrations between 10 mM and 60 mM with the precipitant PEG 2000 MME (polyethylene glycol 2000 monomethylether) between 18% and 21%, again buffered with 100 mM cacodylic acid at pH 6.3.

An initial screen was also carried out with the palmitoleic acid-bound A264E heme domain. Again, 100 mM cacodylic acid at pH 6.3 was used as buffer and the sitting drop technique employed, with crystal growth at 5°C. Palmitoleic acid was added to enzyme to a concentration of 35 μM, corresponding to approximately 6 times the $K_d$, with enzyme again at a concentration of 15 mg/ml. As previously, 2 μl enzyme was added to 2 μl mother liquor.

Crystals were immersed in the cryoprotectant 10% PEG 200, 20 mM MnSO$_4$, 20% PEG 2000 MME, and mounted on nylon loops before being flash frozen in liquid nitrogen. The crystals were maintained in liquid nitrogen until data collection. Data were collected by Dr David Leys and Mr Gordon Joyce (Dept of Biochemistry, University of Leicester) at the ESRF Grenoble synchrotron facility. Structural refinement was also carried out by Dr David Leys and Mr Gordon Joyce for substrate-free and palmitoleic-acid bound A264E heme domain using molecular replacement with the model 1FAG for the substrate-free form and the palmitoleic acid bound form (135).
Chapter 2 Methods

2.16 Hydrostatic pressure spectroscopy

Hydrostatic pressure studies were carried out in INSERM U128, Montpellier in collaboration with Dr Reinhard Lange and in the Department of Biological Sciences, Queen Mary University of London in collaboration with Prof Martin Warren. All hydrostatic pressure studies were carried out using 50 mM MOPS buffer at pH 7.4 (Buffer P) and at 15 °C. For studies of reduced and reduced/CO-bound A264E and wild-type heme domains, buffer P was degassed by extensive bubbling with Argon. Dithionite was then added to saturating concentrations (460 μM). Enzyme concentrations used are specified in Chapter 6. In the case of reduced/CO bound spectra, following addition of enzyme the sample was extensively, but gently, bubbled with CO. Studies of substrate-bound mutant and wild-type heme domains were carried out in the presence of 1 mM arachidonic acid. Following sample preparation, the sample was sealed in a cuvette with a 1 cm pathlength and placed in the pressure cell, which was then sealed and all air displaced with water.

Spectra were recorded using a Cary 3E (Varian) spectrophotometer with a modified cell holder, where the cell was held in a hydrostatic environment. A Haake 3-FQ bath was coupled to the cell holder to allow a constant temperature to be maintained. The pressure was increased in varying increments, detailed in the Results section, and allowed to equilibrate at each step, typically for 5 to 15 minutes. Once no further spectral changes were observed, the pressure was reduced to atmospheric pressure in order to study the reversibility of each process.
Chapter 3  Generation and characterisation of the A264E mutant of flavocytochrome P450 BM3 and its heme domain
Chapter 3 Characterisation of the A264E mutant of P450 BM3

3.1 Introduction

As was described in the introduction (Chapter 1), there has been much interest in the exploitation of P450 BM3 for biotechnological purposes. However, there are a number of factors hindering this, not least the instability of the heme cofactor to dissociation from the protein matrix. It would therefore be of interest and benefit if it were possible to covalently ligate the heme of P450 BM3 to the polypeptide. With this in mind, the A264E mutants of flavocytochrome P450 BM3 and its heme domain were generated and characterised. Previous studies of related CYP4 P450s have demonstrated that a glutamate residue (at the same position as A264 in BM3) forms a stabilizing linkage between the heme macrocycle and the glutamate (193,194).

A number of cytochromes contain covalently linked heme cofactors. For example cytochromes c contain bonds between two cysteine residues and the heme vinyl groups (12). Cytochrome c was first identified in the nineteen twenties by David Keilin, who discovered it through an interest in colour distribution in the tissues of fly larvae. The c-type cytochromes are generally simple electron transfer proteins, shuttling electrons between two other redox centres, with roles in, for example, both the sulphur and nitrogen cycles (12). Mitochondrial cytochromes c generally have histidine and methionine (His-Met) axial coordination to the heme iron, while most others contain bis-histidinyl axial ligation (1). In order for the covalent attachments to form in mitochondrial cytochrome c two additional proteins, known as cytochrome c lyases, are required. It is the sole job of these proteins to assist the formation of the ligations between the heme and cysteines, found in the conserved sequence motif CXXCH, although the mechanism by which this occurs is not understood as yet (195). Bacterial c-type cytochromes utilise a more complex mechanism of covalent attachment, which differs between gram negative and positive bacteria and requires the use of a large number of accessory proteins, the function of many of which has not yet been fully ascertained (16). The reason for this covalent heme attachment has been investigated, but no firm general conclusions can yet be drawn as to whether the linkages are essential for e.g. stability or control of thermodynamic properties. It has been suggested that the reason for this ligation may be to obtain a high ratio of heme to polypeptide (196). For example cytochrome c3 has 4 hemes each surrounded by on
average only 25 residues, a fraction of the number required to stabilise a non-
covalently ligated heme (196). Another suggestion is that, particularly in the case of c-
type cytochromes containing only one heme, the reverse may be true, i.e. that the
covalent ligation is required not to hold the heme in place, but to prevent the
polypeptide from unfolding (196).

Peroxidases are a family of enzymes which utilise hydrogen peroxide to carry out a
number of biosynthetic and degradative functions forming product water and oxygen
(197). Like a number of other mammalian peroxidases, lactoperoxidase contains a
covalently ligated heme. The heme becomes covalently ligated by an autocatalytic
mechanism when hydrogen peroxide is introduced and the non-covalent heme protein
reacts with it, resulting in two ester bonds between an aspartate and the heme 1-methyl
group, and between a glutamate and the heme 5-methyl group (198-200). It has also
been shown that mutating the relevant aspartate and glutamate residues reduces the
amount of covalent heme ligation, and also reduces the enzyme’s activity. Therefore,
the covalent ligations of lactoperoxidase play an important role in the function of the
enzyme (200).

Until recently it has been believed that no cytochromes P450 contain a covalent
ligation to their heme. However it has recently been discovered that a number of
P450s contain a second ligation via the protein to their heme prosthetic group in
addition to the thiol-ligated cysteine residue, which defines P450s. Work by the
groups of Ortiz de Montellano and Rettie has shown that the family 4 P450s studied to
date have a covalent ligation to their heme group (193,194). Family 4 P450s are
mammalian P450s characterised by the capability to hydroxylate the terminal methyl
group of a number of substrates including long chain fatty acids, prostaglandins and
eicosanoids. This hydroxylation is thermodynamically unfavourable and no other
P450 is known which preferentially hydroxylates at the terminal position (201). A
number of the products from this family of enzymes have been shown to be
physiologically active and have therefore been extensively characterised. For example
products of arachidonic acid hydroxylation have been shown to have a role in the
regulation of vascular tone (202). Sequence alignments of the family 4 P450s have
shown that they contain a fully conserved glutamate residue in the I-helix, near the
heme. This residue has been shown to be covalently ligated to the heme in a number of the CYP4 isoforms (193).

In the position occupied by the glutamate in all family 4 enzymes, P450 BM3 has an alanine (A264) and P450 cam a glycine (G248). In fact all other P450s, almost without exception, contain an alanine or glycine at this position (33).

<table>
<thead>
<tr>
<th>CYP</th>
<th>Species</th>
<th>Position</th>
<th>Amino Acid Sequence</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP 102 (B. megaterium)</td>
<td>253</td>
<td>IRYQIITFLIAQHETTSGLLSFALYFLVK</td>
<td>282</td>
<td></td>
</tr>
<tr>
<td>CYP 4A4 (Rabbit)</td>
<td>310</td>
<td>LRAEVDTFMFEGHTTASGTVSWIFYALAT</td>
<td>339</td>
<td></td>
</tr>
<tr>
<td>CYP 4A1 (Rat)</td>
<td>309</td>
<td>LRAEVDTFMFEGHTTASGTVSWIFYALAT</td>
<td>338</td>
<td></td>
</tr>
<tr>
<td>CYP 4B1 (Human)</td>
<td>304</td>
<td>LRAEVDTFMFEGHTTSGISWFLYCNAL</td>
<td>333</td>
<td></td>
</tr>
<tr>
<td>CYP 4C1 (Cockroach)</td>
<td>303</td>
<td>IREEVDTFMFEGHTTASAGCWALLFLGS</td>
<td>332</td>
<td></td>
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<tr>
<td>CYP 4D1 (Drosophila)</td>
<td>305</td>
<td>IREEVDTFMFEGHTTSSALMFYYNIAT</td>
<td>334</td>
<td></td>
</tr>
<tr>
<td>CYP 101 (P. putida)</td>
<td>238</td>
<td>AKRMCGLLLVGLDVTVAFLSFSMEFLAK</td>
<td>267</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.1 Amino acid sequence alignment of the I-helix region of P450 BM3 and P450 cam with other CYP4 enzymes.

A study of members of the CYP4A family has shown that the covalent linkage is formed by an autocatalytic mechanism. Catalytic turnover of the enzyme leads to a vast increase in the proportion of ligated heme (204). It was also shown in the same study that the bond is formed between the 5-methyl group of the heme and carboxyl group of the glutamic acid, probably by the trapping of a transient heme 5-methyl group carbocation by the carboxylic acid group of the glutamate, which is probably formed through activation of the methyl group via the ferryl species (204).
Chapter 3 Characterisation of the A264E mutant of P450 BM3

Figure 3.2 Autocatalytic mechanism believed to be responsible for the formation of the covalent heme ligation in family 4 P450s. Catalytic turnover of the CYP4 enzymes is postulated to produce a transient carbocation species which reacts with the glutamate carboxylate (204).

The structure of P450 BM3 (117,135,142) shows that alanine 264, found to be the equivalent residue to the glutamate of family 4 P450s, with its methyl side chain located in the I-helix and only 5.03Å from the 5-methyl group of the heme for the substrate-bound form of the P450, as shown in Figure 3.3.
Figure 3.3  **Position of the A264 residue in P450 BM3 relative to the heme.**
The alanine is 5.03Å from the heme 5-methyl group. Heme is represented in red, palmitoleate substrate in blue, the proximal ligand cysteine 400 in yellow, alanine 264 in green and the I-helix backbone as a strand (PDB code: 1FAG (135)).

A large amount of protein engineering work has been carried out to change the substrate specificity of P450 BM3 for use in the biotechnology industry. For example Farinas et al. have used a directed evolution approach to generate a variant of P450 BM3 containing 11 amino acid changes, which shows enhanced activity towards alkanes as both an alkane hydroxylase and as an alkane epoxygenase enzyme (205-207). Work has also been carried out into artificial electron donor systems for use with BM3, to obviate the necessity for the expensive pyridine nucleotide reductant NADPH. For example, Cirino and Arnold have mutated P450 BM3 to utilise hydrogen peroxide as electron donor via the peroxide shunt mechanism, as discussed in Chapter 1 (208). A problem for biotechnological exploitation of all P450s is the tendency for them to lose heme, often preceded by loss of native thiolate ligation of the heme iron with P420 formation. Thus, work to create a covalently-stabilised heme in P450 BM3 has great potential for biotechnological exploitation.
This aim of this aspect of my work was therefore to generate the A264E mutation in P450 BM3 and study the effects of this mutation on the enzyme, firstly by testing to see if it has succeeded in introducing a covalent ligation to the heme and then to assess any other changes that may have occurred to the functionality or structure of the enzyme.
3.2 Results

3.2.1 A264E mutagenesis

The A264E mutation was generated in both the heme domain and intact flavocytochrome of P450 BM3. No unique restriction enzyme sites are located at a distance which could be incorporated in a PCR primer for the generation of the A264E mutant using normal PCR mutagenic techniques, so overlapping PCR was employed instead. Three PCRs were used in total, as described in the Methods section. PCRs A and B were successfully carried out, giving DNA fragments of 369bp and 650bp, respectively, when run on an agarose gel. PCR A yielded the DNA fragment from the upstream MfeI restriction enzyme site to slightly downstream of the A264E mutation, PCR B yielded a DNA fragment from slightly upstream of the A264E mutation to the BamHI restriction enzyme site, as detailed in the Methods. The UV irradiation used to locate the ethidium bromide-stained DNA causes thymine cross linking, and therefore should not be used when the DNA is to be excised from the gel and used in further procedures. Instead, a light box was used, where it was not possible to photograph the gel. Remaining PCR product was then run and photographed purely for diagrammatic purposes. PCR C was successfully carried out in the same manner as A and B, generating the combined product of PCRs A and B, i.e. the DNA fragment from the MfeI site to the BamHI site, as discussed in the Methods. A gel of the products is shown in Figure 3.4.
Chapter 3 Characterisation of the A264E mutant of P450 BM3

Figure 3.4 Mutagenic A264E PCR A, B and C products
5 µl of each PCR product was run on a 0.8 % agarose gel against NEB 1 Kb DNA marker, stained with ethidium bromide and photographed under a UV light source. Lanes 1 and 4 show the marker, with the 1 Kb band containing 125 ng DNA, lane 2 shows PCR A product (~ 50 ng) lane 3 PCR B product (~ 800 ng) and lane 5 PCR C product (~ 300 ng).

PCR C was then A-tailed and ligated into pGEM-T, giving the plasmid pGEM-AE. The resultant ligation mix was transformed into JM109 cells and screened by blue/white selection on media plates containing X-gal and IPTG. Plasmid was purified from 5 ml growths of white colonies. Plasmid from 2 of these colonies was then sequenced. DNA sequencing showed that one plasmid contained a number of point mutations, deletions and insertions. The second plasmid contained a second, unwanted, mutation downstream of the desired mutation and upstream of the BamHI restriction enzyme site. Sequence analysis showed that an MscI site was present between the desired and unwanted mutations. MscI cuts to leave a blunt end. It was therefore not selected for original use, since the blunt ended DNA affects the ligation efficiency. The presence of the secondary unwanted mutation, however, necessitated the use of the MscI restriction enzyme site. The plasmid pGEM-AE was digested with MscI and MfeI and, as for the PCR products, this was excised from a 0.8% agarose gel. pBM20 was also digested with MscI and MfeI. Calf intestinal phosphatase was also included in this digest to remove the phosphate groups from the 5’ end to ensure that the plasmid would not ligate back on itself.
Chapter 3 Characterisation of the A264E mutant of P450 BM3

The purified (wild-type) plasmid backbone and (mutant) insert fragments were ligated together. Following transformation into JM109, *E. coli* plasmids were extracted and digested with *BamHI* and *MfeI* together and also with *HindIII*, to verify the ligation had created the correct construct. The results of this are shown in Figure 3.5.

![Figure 3.5 Verification of pGEM-AE plasmid](image)

**Figure 3.5 Verification of pGEM-AE plasmid**

Lane 1 shows 125 ng NEB 1Kb marker, lane two the *HindIII* digested pGEM-AE plasmid, giving bands sized ~230 bp, ~1100 bp and ~3200 bp, lane 3 shows the *MfeI*, *BamHI* digest, giving bands sized ~1000 bp and 3600 bp.

The agarose gel gave the expected results – bands corresponding to the expected sizes of 235 bp, 1134 bp and 3215 bp for the *HindIII* digest, and bands corresponding to 960 bp and 3624 bp for the *BamHI* and *MfeI* digest. The plasmid was sequenced to ensure that the mutation was present using the primers detailed in the *Methods* section. Sequencing was done by automated Sanger dideoxy method at the PNACL facility, University of Leicester and confirmed the presence of the mutation and no further unwanted mutations.

The mutation was also generated in the full length flavocytochrome BM3. Again pGEM-AE was digested with *MfeI* and *MscI* and this fragment ligated into pBM23 (the plasmid encoding flavocytochrome P450 BM3) which had also been digested with *MfeI* and *MscI* and treated with CIP. The ligation product was transformed into JM109 *E. coli* cells. As before, the plasmid A264E pBM23 was verified by DNA sequencing using the plasmids detailed in Chapter 2.
Chapter 3 Characterisation of the A264E mutant of P450 BM3

The purified (wild-type) plasmid backbone and (mutant) insert fragments were ligated together. Following transformation into JM109, *E. coli* plasmids were extracted and digested with *BamHI* and *MfeI* together and also with *HindIII*, to verify the ligation had created the correct construct. The results of this are shown in Figure 3.5.

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82
3.2.2 Over-expression and purification of A264E BM3 enzymes

Heme domain and intact A264E BM3 were both successfully over-expressed and purified using the same protocols as those used to over-express and purify the full length and heme domains of wild-type BM3. Following each round of purification, the enzyme purity was assessed by measuring the ratio of P450 to total protein content. Total protein concentration was taken from the absorbance maximum at 280 nm and the P450 content was determined from the reduced/carbon monoxide-bound adduct. Following the final step of purification, a sample of “pure” protein was run on a SDS-PAGE gel to demonstrate the homogeneity of the sample.

![Figure 3.6](image)

**Figure 3.6** 10 % SDS-PAGE gel showing pure wild-type P450 BM3 heme domain

Shown in lane 1 is a sample of purified wild-type P450 BM3 heme domain (~10 µg) and in lane 2 NEB broad range marker (~2 µg), with band sizes indicated. Lane 1 is seen as a single band corresponding to 54 KDa, showing that the enzyme has been purified to homogeneity.

3.2.3 SDS-PAGE analysis

As was explained in the introduction to this chapter, the A264E mutants were generated to test the hypothesis that insertion of a glutamate, at the position equivalent to that of a fully conserved glutamate in the family 4 P450s, would result in covalent ligation of the BM3 heme to the polypeptide, as occurs in the family 4 P450s. Covalent ligation of heme should result in it remaining bound to the protein backbone.
Chapter 3 Characterisation of the A264E mutant of P450 BM3

under denaturing conditions. This hypothesis was tested by running SDS-PAGE gels of the enzyme in a number of different ligand bound/free forms and redox states, and staining for the presence of heme. Heme and protein stained SDS-PAGE gels of both intact flavocytochrome and heme domains of A264E BM3 are shown below in Figure 3.7 and Figure 3.8.

Figure 3.7 Heme stained (A) and coomassie blue stained (B) 10% SDS-PAGE gels of heme-domain A264E BM3. Lanes 2 and 8 of the gel correspond to NEB broad range protein marker (~2 μg) with band sizes indicated. Lane 1 contains cytochrome c (2 μg), lane 3 A264E P450 BM3 heme domain in the presence of excess laurate, lane 4 A264E heme domain in the presence of excess sodium dithionite, lane 5 A264E heme domain in the presence of both saturating laurate and dithionite, lane 6 A264E heme domain in the presence of hydrogen peroxide and lane 7 oxidised substrate-free A264E heme domain alone (all A264E samples ~25 μg).

Gel A of Figure 3.7 shows only one band containing heme, corresponding to the positive control horse heart cytochrome c. When stained with coomassie blue the gel shows the presence of protein at the correct size for heme domain BM3 in each lane, ensuring that suitable amounts of protein had been loaded to the gel. The different conditions were tested because it had been shown that the covalent ligation in the family 4 P450s occurs by an auto-catalytic mechanism that depends on redox cycling of the enzyme (204), and it was thought that this may also induce ligation of the glutamate on the heme of BM3. The results showed that no covalent ligation is formed to the heme when testing heme domain A264E BM3 either in the isolated form, or following treatment with substrate or reductant. The experiment was repeated using the intact flavocytochrome A264E BM3, to test if the ligation could be formed following enzyme turnover.
Chapter 3 Characterisation of the A264E mutant of P450 BM3

Figure 3.8 Heme-stained and coomassie blue-stained 6 % SDS-PAGE gels of A264E flavocytochrome P450 BM3.
Lanes 4 and 10 contain NEB broad range protein marker (~2 µg), with band sizes indicated to the right of gel B. Lanes 1 and 7 contain flavocytochrome P450 BM3 mutant A264E in the presence of excess NADPH, lanes 2 and 8 contain A264E in the presence of excess NADPH and arachidonate, with lanes 3 and 9 containing A264E with arachidonate, lane 6 contains only A264E (all A264E samples ~ 20 µg). Lane 5 contains the positive control flavocytochrome c (209). All samples were incubated with substrate and/or NADPH for greater than one hour. There is some degradation of the intact A264E P450 BM3, possibly with breakage of the linker resulting in heme domain and flavins domain BM3, the arrow indicates the intact flavocytochrome P450 BM3. The only lane giving a band when stained for heme was the positive control flavocytochrome c, but the coomassie blue stained gel has verified that there is BM3 protein present in all appropriate lanes of the gel.

It can be concluded from Figure 3.8 that covalent ligation between the heme group and polypeptide of A264E P450 BM3 does not occur to any significant extent, even under the enzyme’s true turnover conditions, induced by pre-incubation with the substrate arachidonate and NADPH.

The initial hypothesis behind the creation of this mutant was that it may allow the heme group to become covalently ligated to the A264E residue, and therefore increase the enzyme’s stability. It appears that no such ligation to the protein backbone occurs. Spectroscopic, kinetic and crystallographic characterisation was then carried out to ascertain what, if any, other changes the mutation had brought about.
3.2.4 **Comparisons of wild-type and A264E spectra**

Oxidised and reduced spectra were collected for both the wild-type and A264E mutant proteins, in both the heme domain and the intact flavocytochrome. The heme domain spectra for both wild type and A264E proteins is shown in Figure 3.9, in the oxidised and reduced forms.

![Oxidised and sodium dithionite-reduced spectra of the heme domains of A264E and wild-type BM3](image)

**Figure 3.9** Oxidised and sodium dithionite-reduced spectra of the heme domains of A264E and wild-type BM3

Spectra were collected anaerobically with sample concentration of 6 μM. A wild type oxidised spectrum is shown in black, and the reduced form in blue. An A264E oxidised spectrum is shown in red with the reduced form in green.

Comparisons of the wavelength at which the Soret peak is maximal reveal a ~1 nm shift from 418 nm in the wild type enzyme to 419 nm in the A264E mutant enzyme, with a corresponding shift in the \( \alpha \) and \( \beta \) bands, from 535 nm and 568 nm in the wild-type to 536 nm and 570 nm in the A264E mutant. No difference is seen between the reduced mutant and wild-type enzyme, both giving a Soret peak at 411 nm with fusing of the \( \alpha \) and \( \beta \) bands resulting in single maximum at 550 nm.
3.2.5 **Substrate binding to wild-type P450 BM3 and A264E mutant heme domains**

The saturated C12, C14 and C16 fatty acids laurate, myristate and palmitate, and the unsaturated C16 and C20 fatty acids palmitoleic acid and arachidonic acid were all titrated against the heme domain of the A264E mutant and wild-type P450 BM3. The shift in Soret peak induced on substrate binding was monitored in order to determine dissociation constants ($K_d$ values) for interaction of lipids with wild-type and A264E enzymes, and to assess whether any differences in spectral shift were induced between the two proteins.

**Figure 3.10** **Binding of arachidonic acid to wild-type P450 BM3 heme domain**

The blue spectrum shows ∼5 μM low spin wild-type enzyme and the red spectrum following the addition of 7 μM arachidonic acid. Arachidonic acid was titrated against the enzyme as 0.1 μl aliquots from a 33 mM stock solution (data not shown). Shown in inset is the plot of maximal absorbance change (computed by subtraction of the trough value at 391 nm from the peak value at 423 nm in difference spectra generated by subtraction of the absolute substrate-free spectrum from the appropriate absolute spectrum collected at the relevant point in the titration) against the corresponding arachidonate concentration. Data have been fitted to equation 3 (the quadratic tight binding equation), with the enzyme concentration fixed as 5.2 μM, this gave an $A_{max}$ of 0.14 ± 0.01 and a $K_d$ of 0.55 ± 0.05 μM.

87
Figure 3.11  Titration of arachidonic acid against A264E heme domain.
The initial spectrum, of 7.5 µM A264E heme domain containing no arachidonate, is shown in red. The final spectrum of the titration is shown in blue, and contains 19.6 µM arachidonate. A number of intermediate spectra are shown in black. Arachidonate was added to the enzyme as 0.1 µl aliquots from a 33 mM stock solution in the solvent ethanol. Shown in inset are the difference spectra generated by subtracting the initial spectrum from each successive spectrum in the titration.
Figure 3.12  **Arachidonic acid binding to A264E heme domain.**
Maximal absorbance changes were calculated from the difference spectra as described in the legend to Figure 3.10 (shown in inset in Figure 3.11) and were plotted against the corresponding substrate concentration. These data have been fitted to the tight binding equation (Equation 3), describing the binding of a compound to the enzyme with a \( K_d \) value that is lower than the concentration of protein used for the assay. Fitting the tight binding function generated a \( K_d \) of 0.214 ± 0.019 \( \mu \)M and a \( A_{\text{max}} \) of 0.305 ± 0.004.

Figure 3.11 and Figure 3.12 describe binding of arachidonic acid to the heme domain of the A264E mutant. The binding of arachidonic acid to the A264E heme domain mutant shows a shift in the Soret band from 419 nm to 426 nm on addition of arachidonic acid. Data for the wild type enzyme (shown in Figure 3.10) describe a normal substrate binding type I spectral shift, as expected, and resulting from the change in heme iron d-orbital electron distribution (low-spin to high-spin transition) brought about by the displacement of the water molecule occupying the axial position in the substrate-free enzyme. By contrast, in the A264E mutant the addition of substrate induces a type II spectral shift. This type of shift is generally associated with the binding of an inhibitor to P450 enzymes, with a functional group on the inhibitor ligating directly to the heme at the axial position. This suggests that fatty acids may be...
introducing a ligation-state change of the heme iron in A264E, with the axial water replaced by another ligand.

Titration of the substrates palmitic acid and palmitoleic acid against the A264E mutant also induced a spectral change to 425 nm and 426 nm, respectively giving a type II spectral shift like that seen with arachidonic acid. Titrations of the shorter chain fatty acids laurate and myristate did not give the same extent of substrate-induced shift to a longer wavelength as the longer-chain fatty acids studied. Myristate gave a shift of 1 nm to 420 nm, but laurate did not induce any spectral shift and therefore a binding constant was not calculated for either of these substrates. However, it should be noted that (unlike with wild-type P450 BM3) neither myristate nor laurate produced any spectral change indicative of the formation of high-spin heme iron. The dissociation constants calculated for binding of each substrate to both wild-type and A264E heme domains are given below in Table 3.1.

### Table 3.1 Dissociation constants calculated for substrate binding to wild-type and A264E heme domain BM3.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_d$ (µM)</th>
<th>Wild-type</th>
<th>A264E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laurate</td>
<td>89 ± 15</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Myristate</td>
<td>6.9 ± 0.4</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Palmitate</td>
<td>11.3 ± 0.4</td>
<td>0.045 ± 0.021</td>
<td></td>
</tr>
<tr>
<td>Palmitoleate</td>
<td>3.50 ± 0.17</td>
<td>0.154 ± 0.040</td>
<td></td>
</tr>
<tr>
<td>Arachidonate</td>
<td>0.55 ± 0.05</td>
<td>0.214 ± 0.019</td>
<td></td>
</tr>
</tbody>
</table>

### 3.2.6 Inhibitor binding to A264E

A number of P450 inhibitors, including 4-phenylimidazole, have previously been shown to bind to P450 BM3 and other P450s to give a type II spectral shift characteristic of heme-ligating inhibitor binding to the enzyme (210). Theazole-based P450 inhibitors are able to inhibit the enzyme by binding to the P450 in such a fashion that nitrogen group on an imidazole ring coordinates to the iron in the distal position, displacing the water molecule. The structure of CYP119 has been elucidated with the
inhibitor 4-phenylimidazole bound, clearly showing the nitrogen/iron ligation (211), as is shown in Figure 3.13.

![Figure 3.13](image)

**Figure 3.13 Structure of CYP119 heme with 4-phenylimidazole bound**
The heme of CYP119 is shown in red, with the iron group represented in spacefill and the porphyrin macrocycle as sticks, the proximally ligating cysteine can be seen below the heme. The inhibitor 4-phenylimidazole can be seen above the heme with its imidazole nitrogen ligating to the iron in the distal axial position (211).

The ligation of the azole group nitrogen to the distal position of the heme iron has significant effects on the spectroscopic properties of the enzyme. Figure 3.14 shows the spectral shifts associated with 4-phenylimidazole binding to wild-type P450 BM3 heme domain. These spectral shifts mean that inhibitor binding can provide a valuable spectroscopic characterisation method, enabling e.g. determination of relative P450 affinity for inhibitors of different structural properties, thus helping to define active site geometry.
Figure 3.14 Binding of 4-phenylimidazole to wild-type P450 BM3 heme domain

The spectrum shown in red is of ~5.5 μM wild-type P450 BM3 heme domain prior to the addition of any 4-phenylimidazole. The spectrum is that of a typical low-spin ferric P450 spectrum with Soret maximum at 418 nm. The spectrum shown in blue follows the addition of saturating amounts of 4-phenylimidazole (32 μM), and has a Soret maximum at 424 nm. The relative intensity of the α and β bands is also shifted on 4-phenylimidazole binding. Difference spectra were generated from this data set and the maximal absorbance difference plotted against the corresponding inhibitor concentration (as described above), as shown in the inset. Data are fitted to a rectangular hyperbola (Equation 2), generating a $K_d$ value of 5.8 ± 1.0 μM.
Figure 3.15  Binding of the inhibitor 4-phenylimidazole to A264E BM3 heme domain

4-Phenylimidazole was titrated against 4.6 μM A264E BM3 heme domain in 5 μM additions, each being a 0.1 μl addition from a 50 mM stock solution dissolved in assay buffer. The starting spectrum, collected prior to the addition of any 4-phenylimidazole, is shown in red and has a Soret maximum at 421 nm. The final spectrum collected, corresponding to a 4-phenylimidazole concentration of 130 μM, is shown in blue and has its Soret maximum at 426 nm. A number of intermediate spectra collected during the titration are shown in black.
Chapter 3 Characterisation of the A264E mutant of P450 BM3

Figure 3.16 **Determination of $K_d$ value for 4-phenylimidazole binding to A264E heme domain.**
Following titration of 4-phenylimidazole against A264E BM3 heme domain, difference spectra were generated at each point in the titration and the maximal absorbance change plotted against the concentration of 4-phenylimidazole, as described above. Data were fitted to the tight-binding equation (Equation 3) generating the dissociation constant of 2.51 ± 0.49 µM.

As can be seen in Figure 3.15 and Figure 3.16, binding of 4-phenylimidazole to A264E gives a shift in the Soret band typical of type II inhibitor binding to P450 enzymes, the same as the shift seen with the wild-type enzyme. 4-Phenylimidazole is a relatively small molecule and can penetrate wild-type BM3’s active site easily. The data collected for the A264E mutant indicate that the compound still has high affinity for the mutant active site and binds in the same manner (i.e. with coordination to the heme iron). In order to confirm this, EPR studies have been undertaken in the presence of 4-phenylimidazole. These data are discussed in section 3.2.8.

The small compound sodium cyanide (NaCN) can also be bound to the P450 enzymes giving distinctive spectral shifts. Cyanides are typically weak inhibitors of P450 enzymes. NaCN has been titrated against both wild-type and A264E heme domains of P450 BM3, as shown below.
Sodium cyanide binding to A264E heme domain

0.1 μl aliquots from a 1 M NaCN stock was titrated against ~3.8 μM A264E heme domain. Shown in red is the starting spectrum, which contains no NaCN. The spectrum shown in blue contains 22 mM NaCN, and a number of intermediate spectra are shown in black. Difference spectra were generated as described previously and the maximal absorbance change plotted against the corresponding sodium cyanide concentration, as shown in inset. The apparently sigmoidal data were then fitted to the Hill equation, giving a half-saturating sodium cyanide concentration of 5.4 ± 0.8 mM with a h value of 3.6 ± 1.8.
Figure 3.18  Sodium cyanide binding to wild-type BM3 heme domain.
The red spectrum is for oxidised, NaCN-free enzyme (~ 7 μM enzyme; the protein has some high-spin heme iron content). The blue spectrum is for NaCN-saturated enzyme (4.5 mM cyanide) The black spectra are some of the intermediate spectra collected during the titration. The NaCN-saturated spectrum has its Soret band shifted to 445 nm. Shown in inset are data points generated from difference spectra (as described above) and plotted against the corresponding sodium cyanide concentration. The data have been fitted to a rectangular hyperbola, giving a $K_d$ of 1.68 ± 0.06 mM.

Comparing Figure 3.16 and Figure 3.17 it can be seen that there are substantial differences between the spectra obtained for wild-type and A264E mutant sodium cyanide titrations, suggesting that the molecule is binding in a different manner to the mutant and wild-type enzyme. In the wild-type enzyme the sodium cyanide binds with its carbon group ligating to the heme iron, leading to a final shift in the Soret maximum to 445 nm. The A264E mutant shows spectral changes on binding of sodium cyanide, but the Soret maximum shifts only to 426 nm at saturation, the same wavelength as the Soret maximum shifts to on substrate binding. The absorption shift versus [NaCN] data plot for sodium cyanide binding to the A264E mutant also cannot be fitted to a rectangular hyperbola, describing instead a sigmoid or double (sequential) hyperbola. To approximate the fit, the Hill function was used.
produces a reasonable fit to the A264E data with a positive Hill coefficient value. It is plausible that there is cooperativity in the binding of cyanide to the A264E enzyme and/or that there are conformational rearrangements in the mutant that accompany cyanide binding. This possible allosteric behaviour will be discussed in more detail and in relation to crystallographic data in section 3.2.16.

3.2.7 Redox potentiometry

The redox potential of a P450 can be calculated by monitoring the spectral shift associated with reduction of the P450, and fitting the absorption changes versus the applied potential as described in the Methods section. As was described in the introduction, the redox potential of the P450 enzymes generally becomes more positive on substrate binding. In the substrate-free form of P450 BM3 the d-orbital electrons occupy a low-spin configuration, induced by the presence of a water molecule in the distal axial position on the heme iron. Substrate binding causes displacement of the axial water molecule and a shift in the distribution of the d-orbital electrons to a high spin configuration. These changes are accompanied by a positive shift in the redox potential, such that the first electron can be introduced more easily to the system and enzyme turnover can occur. This system is thought to be one level of control the P450 enzymes have to prevent futile enzyme cycling in the absence of substrate and the accompanied wastage of reducing equivalents in oxygen radical production. The midpoint redox potentials for the one electron reduction of wild-type and A264E heme domain BM3 were calculated in both the substrate-free and arachidonate-bound forms, to monitor any changes in the thermodynamic properties of the heme iron caused by the mutation.
Figure 3.19  Wild-type P450 BM3 substrate-free redox potential
Shown in inset is a fully oxidised spectrum in blue (~8.5 μM) with its Soret maximum at 418 nm. The oxidised sample was sequentially reduced and several spectra collected at various applied potentials (data not shown). The fully reduced spectrum is shown in red with its absorbance maximum at 410 nm. The absorbance change at 404 nm (a point near the maximal overall change in absorption between oxidised and reduced forms) was plotted against the corresponding potential (main figure). The data are fitted to the Nernst single electron equation, giving a redox potential for the reduction of substrate-free wild-type P450 BM3 heme iron of -392 ± 5 mV.
UV-visible absorption spectra accompanying reduction of A264E heme domain, and calculation of the reduction potential

The starting spectrum, shown in red is that of fully oxidised A264E heme domain with its Soret maximum at 419 nm. The enzyme was progressively reduced with dithionite and the spectra shown in black are a selection of those for the partially reduced intermediate species. The spectrum displayed in blue shows the enzyme in the fully reduced (ferrous) state, with a Soret maximum at 408 nm. A plot of absorption change at 408 nm versus the corresponding redox potential is shown in inset, and the resultant data are fitted to a one electron Nernst equation giving a midpoint potential of -316 ± 5 mV.
Figure 3.21 Reduction of substrate-bound wild-type P450 BM3 and calculation of redox potential
The spectrum for 9.5 μM wild-type P450 BM3 heme domain fully complexed with arachidonic acid is shown in red. The P450 has a typical high-spin spectrum with its Soret maximum at 391 nm. The spectrum shown in blue is the final reduced spectrum of the P450, collected after complete conversion of the P450 to its ferrous state by addition of excess dithionite. This spectrum displays typical features of a reduced P450, with a Soret maximum at 410 nm and fused α and β bands with maximum near 550 nm. Shown in black are selected intermediate spectra collected at various points during the reductive titration. The absorbance at 408 nm was plotted against the corresponding potential and the data fitted to a single electron Nernst equation, generating a redox potential of -283 ± 5 mV, as shown in inset.
Figure 3.22 Reduction of arachidonate-bound A264E heme domain and calculation of redox potential

Reduction of substrate-bound P450 (~14 μM, red spectrum) with sodium dithionite results in a shift of the heme Soret maximum from 425 nm to 409 nm (blue spectrum). The absorbance values at 409 nm were plotted against the corresponding potential (shown in inset) and fitted with the Nernst single electron equation, yielding a midpoint potential of -314 ± 2 mV.

Wild-type redox potentiometry (Figure 3.19) gave a substrate-free potential of -392 ± 5 mV and an arachidonate-bound potential of -283 ± 4 mV, i.e. a positive shift of ~109 mV on substrate binding. These data are in good agreement with those observed in earlier studies of wild-type and F393 mutants of P450 BM3 involving redox potentiometry (100,153).

The substrate-free and arachidonate-bound A264E redox potentials have produced thermodynamic data which are not significantly different from one another. This suggests that substrate does not influence the potential of the heme in the same manner seen for wild-type enzyme. Binding of substrate does not appear to induce the normal elevation of heme iron reduction potential seen with the wild-type enzyme. As was
discussed in section 1.4.2 of the *Introduction*, substrate binding causes the d-orbital electrons of the ferric P450 heme iron to re-distribute, resulting in a shift of heme iron spin-state equilibrium from low- to high-spin, elevating the redox potential such that it is favourable for the introduction of the first electron. No change in the redox potential in the presence of substrate suggests that there may be no change in spin-state. In the substrate binding titrations on A264E shown above in section 3.2.5, a type II spectral shift was observed, which usually corresponds to the binding of a ligand to the heme iron. The data thus far are consistent with the A264E heme remaining in a low-spin form on substrate binding, explaining the apparent lack of perturbation of redox potential. The redox potential of the A264E mutant is significantly different from that of the substrate-free wild-type enzyme, suggesting that the mutation causes changes other than maintaining the enzyme in a form similar to that of the substrate-free wild-type enzyme throughout. If this was the case, it might be expected that the redox potentials measured for A264E would be closer to that of the substrate-free wild-type enzyme (rather than ~75 mV more positive).

### 3.2.8 EPR

Electron paramagnetic resonance (EPR) spectroscopy reports on the paramagnetic species contained within a sample, *i.e.* on the systems present that contain unpaired electrons. In the case of redox enzymes, EPR can be used to study the redox-active centre, since this is usually the only paramagnetic region of the enzyme. In the case of P450s, the ferric heme iron provides a distinctive and informative EPR spectrum that reports on coordination state of the heme iron. From the spectrum obtained, a g-value, or a number of g-values can be calculated, which are affected by the orientation of the molecule in a magnetic field, and therefore report on the environment of the molecule (212). EPR was used to study the heme iron coordination state of the A264E BM3 heme domain in the substrate-free, substrate-bound and inhibitor-bound states, as a way to further interpret the unusual results seen in substrate binding studies.

EPR spectra were collected and analysed for A264E heme domain in the substrate-free, arachidonate-bound and 4-phenylimidazole bound forms in collaboration with Dr
Myles Cheesman (Department of Chemistry, University of East Anglia, Norwich) and the results obtained were compared to those obtained in earlier studies.

![EPR spectra of substrate-free and arachidonate-bound forms of the wild-type and A264E heme domain]

**Figure 3.23** EPR spectra of substrate-free and arachidonate-bound forms of the wild-type and A264E heme domain

EPR spectra were collected with a protein concentration of ~400 μM, and with arachidonate concentration of 500 μM. Saturation with substrate was confirmed by recording optical spectra prior to collection of EPR spectra. The substrate-free wild-type spectrum is shown in red and arachidonate-bound shown in blue, the substrate-free A264E spectrum is shown in green and the arachidonate-bound form in black, g-values are indicated on each spectrum.

Earlier studies of wild-type BM3 have shown that its g-values in the substrate-free, ferric, low spin form are 2.42 (g_x), 2.26 (g_y) and 1.92 (g_z) (140), these are in agreement
with those observed in Figure 3.23 (and summarised in Table 3.2). As can be seen in Figure 3.23, the g-values of the substrate-free A264E mutant are seen at 2.56 \( (g_z) \), 2.45 \( (g_y) \), 2.26 \( (g_x) \), 1.91 \( (g_z) \) and 1.85 \( (g_x) \). There is therefore some heterogeneity in the sample. Dawson et al. (139) have carried out an extensive study of P450 cam ligand complexes using UV-visible absorbance, MCD and EPR spectroscopy in order to characterise the species. A variety of simple ligands were chosen with functionalities to mimic a number of amino acids. For example, indole was used to study tryptophan ligation and imidazole to study histidine ligation. Comparisons of the EPR data obtained by Dawson et al. (139) with data obtained for the substrate-free A264E BM3 mutant concur with there being partial heme iron ligation by a molecule that mimics ligation by acetate or formate in the P450 cam enzyme. The rest of the molecules in the substrate-free A264E sample have g-values that are near-identical to those for the substrate-free wild-type P450 BM3, indicating that the remaining molecules have typical P450 cysteinate and water axial ligands to the heme iron. There was no acetate or formate (or other carboxylate-containing molecule) in the A264E sample used. However, the A264E mutation introduces an acidic amino acid side chain in the environment of the heme. Thus, the data suggest that the EPR spectrum observed for substrate-free A264E results from a novel Glu-Fe-Cys heme iron ligand set in a proportion of the enzyme (with the remainder having water-Fe-Cys ligation).

On saturation with arachidonate, the g-values for A264E are shifted to 2.56 \( (g_z) \), 2.45 \( (g_y) \), 2.26 \( (g_x) \), 1.92 \( (g_z) \) and 1.88 \( (g_x) \), although with a much stronger contribution from the 2.45 and 1.92 g-tensor components than from those at 2.56 and 1.88. These values correlate with a shift in the equilibrium almost entirely to the proposed glutamate-ligated form. No g-values typical of a high spin component are seen by EPR with substrate-bound enzyme. The spectral shift seen on substrate binding in the UV-visible absorption region is therefore likely due to the substrate causing a larger proportion of the heme iron in A264E to adopt glutamate ligation. Thus, EPR data are consistent with partial coordination of the A264E heme iron by the E264 side chain in the substrate-free form, with arachidonate binding leading to almost complete coordination by the glutamate at the expense of the water-ligated species observed in the substrate-free form.
Figure 3.24 4-Phenylimidazole bound EPR spectra of A264E and wild-type heme domain

EPR spectra were collected of ~ 400 μM enzyme following the addition of ~ 2 mM 4-phenylimidazole. Wild-type is shown in red and A264E in green. UV-visible spectra were collected to ensure saturation with 4-phenylimidazole prior to collection of EPR spectra. g-values are indicated on both spectra.

In the wild-type enzyme some heterogeneity is seen in the wild-type complex with minor signals at 2.44 and 1.92 possibly indicating unligated enzyme which still retains normal distal water ligation, there also appear to be. The addition of 4-phenylimidazole to A264E results in one dominant species with g-values of 2.58 (g_z), 2.26 (g_y), 1.86 (g_x). These values correlate with data obtained for the wild-type enzyme showing that 4-phenylimidazole is ligating to the mutant enzyme in the same manner as to the wild-type enzyme with the nitrogen ligating directly to the heme iron.

<table>
<thead>
<tr>
<th></th>
<th>g_z</th>
<th>g_y</th>
<th>g_x</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>2.42</td>
<td>2.26</td>
<td>1.92</td>
</tr>
<tr>
<td>Wild-type + Substrate</td>
<td>8.18</td>
<td>2.26/3.44</td>
<td>1.66</td>
</tr>
<tr>
<td>Wild-type + 4 PheIm</td>
<td>2.57</td>
<td>2.26</td>
<td>1.86/1.85</td>
</tr>
<tr>
<td>A264E</td>
<td>2.57/2.45</td>
<td>2.26</td>
<td>1.91/1.85</td>
</tr>
<tr>
<td>A264E + Substrate</td>
<td>2.56/2.45</td>
<td>2.26</td>
<td>1.92/1.88</td>
</tr>
<tr>
<td>A264E + 4PheIm</td>
<td>2.58</td>
<td>2.26</td>
<td>1.86</td>
</tr>
</tbody>
</table>
3.2.9 MCD

MCD is defined as the differential absorption of left and right plane polarized light when the sample has a magnetic field applied parallel to the light beam (213). Circular dichroism (CD) spectra collected of samples report on their chirality, by nature of the fact that left and right circularly polarized light are chiral to one another and therefore differentially absorbed to one another by chiral molecules. CD spectra are therefore widely used to report on secondary structure of protein or DNA (214,215). The inclusion of a magnetic field to the sample means that the resultant spectra report on the differential absorption of left and right plane polarized light due to the effect of the applied magnetic field. The applied field affects the degeneracy of electrons, splitting them further and therefore increasing the difference between the absorption of the left and right circularly polarized light – this is termed the Zeeman effect (213). MCD therefore provides a sensitive method for analysis of the heme iron and its ligands. MCD has been used here in both the near-infrared and UV-visible regions to analyse the substrate-free and substrate-bound wild-type P450 BM3 and its A264E mutant. Spectra were collected in collaboration with Dr Myles Cheesman (Department of Pure and Applied Chemistry, University of East Anglia).
Chapter 3 Characterisation of the A264E mutant of P450 BM3

Figure 3.25 Wild-type and A264E heme domain near infrared MCD spectra

Spectra were collected for ~300 μM wild-type and A264E heme domain proteins in a 1 mm pathlength cuvette. Shown in black is the wild-type substrate-free spectrum (300 μM), with its spectral maximum at 1078 nm. Substrate bound wild-type BM3 (550 μM) is shown in red with its spectral maximum shifted to 834 nm. Shown in green is the substrate-free A264E mutant (690 μM) and in blue substrate-bound A264E (660 μM), both with spectral maxima at 1084 nm. Substrate (arachidonate) was added to protein solutions progressively to a final concentration of 1 mM. The predominantly high-spin signal observed for the wild-type P450 indicates that the majority of this sample is in the substrate-bound form.

The near-infrared (NIR) MCD spectra of wild-type and A264E heme domain shown in Figure 3.25 show typical low spin, ferric P450 in the substrate-free form of both proteins. Addition of substrate to the wild-type enzyme gives a large change in the spectral maximum from 1078 nm to 834 nm typical of the predominance of a high-spin P450 and in close agreement with those data seen for P450 cam (216). Addition of substrate to the A264E mutant did not cause the same shift to a spectrum typical of high spin P450, but instead has shown little change, indicating again that no high spin P450 is formed on addition of substrate to the A264E mutant. These data are consistent with the results from optical titrations and from EPR studies. A slight shift in spectral maximum is seen between the wild-type and A264E mutant (from 1078 nm to 1084
nm). This can be attributed to the presence of the glutamate as distal ligand in a large proportion of the A264E mutant species. The absolute position of the NIR MCD maximum is a powerful diagnostic tool for identifying heme iron coordination state. The difference between the wild-type and A264E maxima is thus a consequence of the change between aqua- and carboxylate- (glutamate-) ligands at the distal position on the heme iron.

![Near Infrared MCD Spectra](image)

Figure 3.26 Wild-type and A264E mutant BM3 heme domain near UV-visible MCD spectra

Wild-type P450 BM3 heme domain is shown in black (30 μM) and in red (45 μM) following the addition of the substrate arachidonate (250 μM). The A264E mutant is shown in green (33 μM) and in blue (63 μM) following the addition of the same final concentration of arachidonic acid.

As with the near infrared spectra, the wild-type enzyme has a spectrum typical of low-spin ferric P450 prior to the addition of substrate, and signals indicative of high-spin ferric heme are seen following the addition of substrate. In the low-spin form, peaks and troughs are seen at 409 nm (peak, p), 429 nm (trough, t), 521 nm (p), 540 nm (t), 559 nm (p) and 575 nm (t) and are all indicative of a low-spin ferric thiolate-ligated heme iron (164). The addition of substrate to the wild-type enzyme is seen as a change in intensity with an increase in the charge transfer band seen at 655 nm, new spectral features between 360 nm and 405 nm and a shoulder at 555 nm, all of which are
indicative of a shift towards a high-spin P450. The A264E mutant also displays a spectrum typical of low-spin ferric heme prior to the addition of substrate, and shows no major changes on addition of substrate.

The MCD data are in close agreement with other spectroscopic data collected, showing that no significant amount of high-spin P450 is seen on addition of substrate to the A264E mutant. The changes in the near-infrared MCD region observed between A264E and the wild-type BM3 are almost certainly due to the presence of the E264 residue ligating to the heme iron. Thus, these data can provide fingerprint information for the analysis of novel heme proteins suspected of having new ligation states.

**3.2.10 Resonance Raman**

Resonance Raman spectroscopy is a powerful method for studying the vibrational frequencies of specific bonds within a compound. The vibrational frequencies and intensities are sensitive to the structure and local environment of the atoms involved, allowing assignation of particular signals to particular bond stretches or other deformations (217). Through laser irradiation at 406 nm (a wavelength available on the laser apparatus used and one close to the Soret maximum of the P450s), vibrational information specific to the heme chromophore can be obtained. Resonance Raman studies were carried out on the A264E heme domain to examine the effect on heme vibrational signals that occur due to the unusual heme ligation found in the mutant.

Resonance Raman spectra were collected in collaboration with Dr Rachael Littleford (Department of Chemistry, University of Strathclyde, Glasgow).
Figure 3.27  A264E and wild-type P450 BM3 heme domain resonance Raman spectra
Spectra were collected with 50 μM enzyme using 406 nm excitation. Shown in red is the substrate-free A264E spectrum and in blue the wild-type BM3 spectrum. The major band observed is that for the oxidation state marker, \( \nu_A \), indicating that the enzymes are in the ferric form.
Characterisation of the A264E mutant of P450 BM3

Figure 3.28 Wild-type P450 BM3 and A264E high-frequency resonance Raman spectra
Shown in red is the substrate-free A264E mutant spectrum and in green the substrate-free wild-type spectrum both at a concentration of 50 μM. Spectra show more detail of the high frequency region seen in Figure 3.27. Curve fitting carried out from the A264E mutant is shown in black and residual spectrum from subtraction of the curve fit from the original spectrum shown in blue. Assignments made on the basis of the curve fit data and work by Smith et al. are indicated (218).

The substrate-free wild-type and A264E resonance Raman spectra are quite similar to one another with very few changes seen in the entire spectrum or on closer examination of the high-frequency region.

The \( v_4 \) band of the spectrum reports on the oxidation state of the enzyme (219). In both the substrate-free and arachidonate-bound forms of the A264E enzyme the \( v_4 \) band is at 1371 cm\(^{-1}\), indicating that the enzyme remains in the ferric form, as is expected. The \( v_3 \) band reports on spin state, which also influences the \( v_{36}, v_{11}, v_2, v_{37} \) and \( v_{10} \) markers (219). None of these bands change significantly on substrate
Chapter 3 Characterisation of the A264E mutant of P450 BM3

(arachidonate) binding (not shown) and their position confirms the low-spin state of the A264E enzyme both plus and minus substrate. By contrast, a significant shift towards the high-spin form is seen with fatty acid additions to the wild-type P450 BM3 enzyme. This result again confirms that the low-spin form of the heme iron remains predominant in both substrate-free and substrate-bound forms of A264E, as was also observed by EPR and inferred from optical substrate binding titrations. No specific evidence for glutamate ligation is obtained from the resonance Raman studies. This is possibly due to changes in axial ligand from water to glutamate occurring as out-of-range heme vibrational modes, which are not resonantly enhanced with excitation at 406 nm. Resonance Raman of hemes is more sensitive to in-plane vibrations of the cofactor. Thus, it is not surprising that it as proven an inferior tool to EPR and MCD in the characterization of changes in axial ligands.

3.2.11 Steady-state kinetics

Steady-state kinetic measurements were made to assess the substrate-dependent NADPH oxidation by wild-type and A264E flavocytochromes P450 BM3, using the same substrates as those used in optical binding titrations. Since binding of substrate does not give any apparent shift in the redox potential of the A264E mutant, and since the E264 side chain ligates to the heme iron in substrate-dependent manner, it follows that there may be substantial changes to the steady-state kinetic behaviour of the mutant enzyme with respect to wild-type P450 BM3.

Each of the substrates tested resulted in A264E enzyme activity, but the rates were significantly reduced when compared to those obtained for the wild-type enzyme.
Chapter 3 Characterisation of the A264E mutant of P450 BM3

Figure 3.29 Palmitoleic acid-dependent NADPH oxidation by the A264E mutant of flavocytochrome P450 BM3.
Rates from three assays were averaged at each palmitoleic acid concentration, and resultant data fitted to the Michaelis-Menten function. The $k_{cat}$ was calculated to be $3090 \pm 90 \text{ min}^{-1}$ and the $K_M = 1.7 \pm 0.2 \mu M$.

Table 3.3 $k_{cat}$ and $K_M$ values obtained as substrate dependent NADPH oxidation with wild-type and A264E BM3.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Wild-type $k_{cat}$ (min$^{-1}$)</th>
<th>$K_M$ (µM)</th>
<th>A264E mutant $k_{cat}$ (min$^{-1}$)</th>
<th>$K_M$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laurate</td>
<td>$2770 \pm 120$</td>
<td>$87.4 \pm 8.1$</td>
<td>$52 \pm 2$</td>
<td>$133 \pm 14$</td>
</tr>
<tr>
<td>Myristate</td>
<td>$4835 \pm 295$</td>
<td>$37.3 \pm 11.5$</td>
<td>$70 \pm 2$</td>
<td>$10.9 \pm 1.3$</td>
</tr>
<tr>
<td>Palmitate</td>
<td>$4590 \pm 407$</td>
<td>$11.5 \pm 4.9$</td>
<td>$915 \pm 80$</td>
<td>$9.95 \pm 0.8$</td>
</tr>
<tr>
<td>Palmitoleate</td>
<td>$6980 \pm 430$</td>
<td>$0.75 \pm 0.25$</td>
<td>$3090 \pm 90$</td>
<td>$1.7 \pm 0.2$</td>
</tr>
<tr>
<td>Arachidonate</td>
<td>$16400 \pm 185$</td>
<td>$5.1 \pm 0.43$</td>
<td>$1709 \pm 119$</td>
<td>$0.64 \pm 0.143$</td>
</tr>
</tbody>
</table>

As can be seen in Table 3.3, the rates of substrate-dependent NADPH oxidation are significantly decreased in the A264E mutant when compared the wild-type enzyme for all substrates tested. However, the fact that the mutant retains oxidation activity suggests that, despite the apparent substrate-induced heme iron ligation observed from optical titrations and other spectroscopic analyses, the ligand may be displaced from the iron following electron transfer from the reductase redox partner, enabling a significant amount of enzyme turnover, particularly with the longer chain substrates.
3.2.12 Turnover studies

In addition to studying the steady-state kinetics of the A264E enzyme, product and substrates have also been studied by mass spectrometry. This was of particular importance to establish whether the substrate-dependent NADPH oxidation observed in steady-state analysis was linked to fatty acid oxygenation. Enzyme was incubated for 12 hours with 45 µM lauric acid and 600 µM NADPH. Following turnover, products and remaining substrate were isolated and samples analysed by mass spectrometry, as described in the Methods section. Turnover experiments were carried out with both wild type and A264E flavocytochrome P450 BM3. The data are shown in Figure 3.30 and Figure 3.31.

Figure 3.30 Mass spectrometry analysis of lauric acid turnover by wild-type flavocytochrome P450 BM3.
The substrate, lauric acid, has a mass of 199 amu. The product, mono-hydroxylated laurate, has mass of 215 amu. The mass spectrum therefore shows that all the substrate has been converted to mono-hydroxylated product with no detectable amount of the substrate remaining.
Figure 3.31 Mass spectrometry analysis of lauric acid turnover by A264E flavocytochrome P450 BM3
A small amount of mono-hydroxylated product can be seen with a mass of 215 amu, with the principal feature being the substrate lauric acid with a mass of 199 amu. Small amounts of contaminants are also seen at higher m/z values.

Comparing Figure 3.30 and Figure 3.31 it can be seen that both the wild-type and A264E mutant form product from laurate when incubated in their flavocytochrome form with substrate and NADPH, but that the quantity of product formed is significantly less with the A264E mutant than with the wild-type enzyme. The incubation with NADPH was for an adequate time such that full consumption of NADPH occurred. Although laurate is the weakest binding of all substrates tested it has the highest solubility without requirement for solvents which adversely affect the reaction, and is therefore the best substrate used for turnover assays. This was verified by UV-visible absorbance spectra, which showed that the typical UV-visible absorption spectrum of NADPH (with maximum at 340 nm) had disappeared after the incubation. In view of the possibility that the A264E mutant catalyses predominantly the uncoupled oxidation of NADPH, producing water, hydrogen peroxide and/or superoxide as described in section 1.4.7 of the introduction, studies of the production of peroxide were carried out.
3.2.13 Measurement of uncoupled turnover

As has been demonstrated in steady-state and turnover studies, the A264E mutant is capable of substrate-dependent NADPH consumption, but full conversion of laurate to hydroxylated product was not observed in turnover studies. It is therefore possible that a quantity of the NADPH is being oxidised to form water, hydrogen peroxide and/or superoxide, which can all be produced when the enzyme turns over in an uncoupled manner. It is possible to measure formation of hydrogen peroxide and superoxide in a coupled assay with the enzymes horseradish peroxidase (HRP) and superoxide dismutase (SOD), and by using the peroxidase substrate o-dianisidine. The overall reactions catalysed by HRP and SOD are shown in figure 3.32.

\[
\begin{align*}
A. & \quad \text{H}_2\text{O}_2 + 2\text{HS} \rightarrow 2\text{H}_2\text{O} + 2\text{S}^* \\
B. & \quad 2\text{O}_2^{-} + 2\text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2
\end{align*}
\]

**Figure 3.32** HRP and SOD overall reactions catalysed

Reaction A shows the overall reaction catalysed by HRP, breaking down hydrogen peroxide to 2 water molecules with 2 additional protons coming from the substrate (S), which is also oxidised in the reaction. Reaction B shows the overall reaction catalysed by SOD, forming 1 oxygen molecule and 1 hydrogen peroxide molecule from 2 superoxide molecules and 2 protons.

The compound o-dianisidine acts as substrate for HRP, it is a colourless compound when reduced but oxidation causes it to become red in colour. HRP has a very high activity so it can be assumed that all hydrogen peroxide in the assay is converted to water with the corresponding oxidation of o-dianisidine. Measuring the reduction of o-dianisidine can therefore allow quantification of the hydrogen peroxide in the assay. NADPH was added to assays containing enzyme in assay buffer, assays were set up both with and without the substrate laurate. The NADPH was all consumed before the addition of HRP and o-dianisidine, and the absorbance increase at 440 nm monitored. In assays to monitor superoxide formation SOD was added to the assay prior to the addition of NADPH, where it converts superoxide to hydrogen peroxide, which is then broken down by the HRP. Shown below in Table 3.4 are the percentages of NADPH utilised to form hydrogen peroxide or superoxide. These results are discussed below.
Table 3.4 Percentages of NADPH converted to hydrogen peroxide or superoxide in wild-type and A264E mutant flavocytochrome P450 BM3

200 μM NADPH was incubated with ~ 0.2 μM enzyme in the presence and absence of 950 μM laurate. Assays with and without laurate were begun simultaneously and measurements made once all NADPH was oxidised in assays containing substrate.

<table>
<thead>
<tr>
<th>% NADPH converted to:</th>
<th>Wild-type</th>
<th>Wild-type + Laurate</th>
<th>A264E</th>
<th>A264E + Laurate</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O₂</td>
<td>3.3 %</td>
<td>1.7 %</td>
<td>3.2 %</td>
<td>26 %</td>
</tr>
<tr>
<td>Superoxide</td>
<td>1.2 %</td>
<td>4.1 %</td>
<td>4 %</td>
<td>40 %</td>
</tr>
</tbody>
</table>

As can be seen in Table 3.4, and as has been show by Noble et al (76) previously, there is very little uncoupling in the wild-type enzyme, with the majority of the NADPH being used to generate product rather than superoxide or hydrogen peroxide. In the absence of fatty acid substrate, the enzyme slowly oxidises some NADPH through its reductase domain with production of superoxide/peroxide (220). The data obtained in the absence of substrate for the wild-type enzyme do not account for all NADPH being consumed, some is possibly lost to form water, with some NADPH oxidised in the air environment without reacting with the enzyme. Unlike the wild-type enzyme, in the presence of laurate, the A264E mutant oxidises a large proportion of NADPH to form hydrogen peroxide and superoxide, rather than to form hydroxylated product. It is possible that due to the elevated potential of the heme observed for A264E in both substrate-free and substrate-bound forms (relative to wild-type substrate-free enzyme) there is a greater “leak” of electrons to the P450 heme iron in the laurate-bound A264E than in laurate-bound wild-type P450 and that this is a major factor underlying the greater level of uncoupling of NADPH oxidation from lauric acid oxygenation in this mutant.

3.2.14 Circular Dichroism

Circular dichroism (CD) spectroscopy can be used to study enzyme secondary and tertiary structure, and examine the chirality of any redox centres or other cofactors (assuming that these absorb light at longer UV-visible wavelengths from those at
which aromatic amino acids and secondary structural elements absorb). CD spectra report on the differences in absorption of left and right circularly polarised light by a chiral compound. Spectra collected in the far UV region (~190-260 nm) report on the secondary structure of the protein. In the near UV region (~260-320 nm) CD absorption is primarily due to aromatic residues and reports on enzyme tertiary structure. In the visible region (at longer wavelengths) CD absorption by a P450 enzyme is predominantly due to its heme group, and is sensitive to the redox state and presence of ligands or substrates (214,215). CD spectra were collected for the A264E BM3 mutant to assess any structural changes when compared to the wild-type enzyme, and to look at the changes in the heme environment on substrate or inhibitor binding to the enzyme.

Circular dichroism spectra were collected for the A264E heme domain in the substrate-free, arachidonate-bound and 4-phenylimidazole-bound states, in both the near UV - visible (vis-NUV) region and the far UV (FUV) region.

![CD spectra](image)

**Figure 3.33** Far UV CD spectra of A264E heme domain
Substrate-free A264E heme domain at a concentration of 1.8 µM is shown as a black line, arachidonate-bound (20 µM) as a red line and 4-phenylimidazole bound (15 mM) as a green line.

FUV spectra show no major changes in secondary structural properties of the A264E heme domain in the presence of the inhibitor ligand 4-phenylimidazole or the substrate arachidonate. The substrate-free, substrate-bound and inhibitor bound spectra all show
the same basic shape, indicative of a predominantly helical protein. The lack of major secondary structural change induced on substrate or ligand-binding is consistent with CD data for the wild-type enzyme and with structures of the substrate-free and palmitoleate-bound wild-type P450 BM3 (135, 142, 221).

![Visible and near UV CD spectra of A264E AFD](image)

Substrate-free enzyme (45 µM) is shown in black, the arachidonate-bound form in red (150 µM) and the 4-phenylimidazole-bound form (1 mM) in green.

The visible-near UV spectra of A264E heme domain show changes with binding of substrates and ligands. The NUV region of the spectrum reports on the aromatic residues, so changes seen with 4-phenylimidazole may be mainly due to the inhibitor itself, rather than to any significant change in the enzyme tertiary structure. Changes in the visible region of the spectra report on the heme. Binding of 4-phenylimidazole and arachidonate induces different spectral perturbations in this region. This suggests that the two molecules bind to the enzyme to induce subtly different changes to the chirality and/or electronic properties of the heme, although they give almost exactly the same UV-visible spectral changes. The states of the enzyme were verified by UV-visible spectroscopy prior to the CD spectra being collected, to ensure ligand saturation. The most obvious difference between the arachidonate- and 4-phenylimidazole-bound visible CD spectra is in the relative intensity of the Soret CD band (which has a negative sign in all cases, but has a substantially lower CD extinction coefficient in the case of the 4-phenylimidazole-bound form than in the cases of the substrate-free or arachidonate-bound forms). A second negative CD signal
is seen in the vicinity of the P450's δ-absorption band (at ~350 nm), and in this case is of greater CD extinction coefficient in the case of the 4-phenylimidazole-bound form than for the other two species.

### 3.2.15 Denaturation

A number of denaturation studies were carried out with the A264E mutant of P450 BM3 in order to assess whether the mutation affects the stability of secondary and tertiary structure, or the binding of the heme to the protein backbone. It was of interest to examine whether the altered coordination of the heme iron led to any changes in the stability of the A264E mutant. Further, effects of pH and ionic strength were examined on the heme iron coordination state in the A264E mutant. Since, in absence of substrate, there appears to be only partial ligation of E264 to the heme iron, it was of interest to examine whether these changes in physical parameters might have effects in perturbing heme iron coordination or promoting further the ligation of the glutamate ligand to the heme iron.

#### 3.2.15.1 Thermal effects on P450 spin-state and structural stability

The effects of temperature were studied on the A264E heme domain. It has previously been shown that the spin-state equilibrium in P450 cam can be shifted from low- towards high-spin by increasing the temperature of the protein environment (222). The addition of fatty acid substrate does not cause the "normal" shift from low- to high-spin with the A264E BM3 mutant; instead EPR and other spectroscopic data suggest that binding of long chain fatty acid substrates induce glutamate ligation to the iron in A264E. Effects of temperature change were therefore studied with the A264E BM3 heme domain, with the hypothesis that altering the temperature might cause some changes in the spin state of the heme iron, or else induce further changes in the coordination of the heme iron by E264.
Figure 3.35 **Effects of temperature on wild-type P450 BM3 heme domain**

Spectra were collected for ~8 μM BM3 heme domain at temperatures between 20 and 60 °C in 5 °C increments. The spectrum shown in red was collected at 20 °C, and the one in blue at 60 °C, spectra shown in black were collected at intermediate temperatures (30 °C, 40 °C, 50 °C).
Figure 3.36  **Effect of temperature on A264E heme domain.**
The starting spectrum of substrate-free A264E heme domain (~ 4.5 μM) was collected at 6 °C, and is shown in red. The final spectrum, collected at 60 °C, is shown in blue. A number of spectra collected at intermediate temperatures are shown in black.

Figure 3.35 and figure 3.36 show the effects of temperature on wild-type BM3 heme domain and the A264E BM3 heme domain mutant. The starting spectrum of the wild-type enzyme (20 °C) has the Soret band at 418 nm while the Soret band of the substrate-free A264E mutant (6 °C) is found at 419 nm. These values are consistent with the preceding studies for both wild-type and the A264E mutant. The wild-type enzyme shows a small degree of conversion towards the high-spin form, as has been shown with P450 cam (222). In wild-type BM3, there is a small decrease in the Soret maximum at 418 nm as the temperature is elevated, concomitant with the development of absorption at ~ 390 nm, which is indicative of the development of the high-spin form. An isosbestic form is observed at ~405 nm indicates that there is a relatively simple equilibrium between low-spin and high-spin species observed for wild-type P450 BM3 as temperature is changed. By contrast, there is a progressive decrease in the intensity of the UV-visible spectrum of the A264E mutant in the temperature range up to ~ 45 °C. The spectrum remains similar in form to that of the starting spectrum in this regime, suggesting that there is no major change in the coordination state of the A264E heme in those molecules that provide the Soret signal in this range. At higher temperatures (above ~ 45 °C) a broad band centred at 375 nm appears, probably
indicative of loss of heme from the protein matrix. At no point in the experiment do signals accumulate that are typical of the increased formation of further glutamate-ligated heme iron (*i.e.* with an absorption maximum shifting towards 426 nm) or of increased formation of high-spin heme iron as is seen in wild-type. It therefore appears that, unlike wild-type BM3, temperature elevation does not induce increases in high-spin contact of the A264E mutant, or any significant increase in the proportion of molecules in which E264 is ligated to the heme iron. However, the large decreases in Soret intensity (up to ~45 °C) and further shifts observed up to 60 °C suggest that the A264E mutant is structurally less stable than is the wild-type BM3, with large amounts of heme dissociation from the 264E protein matrix occurring across the same temperature range as that which causes only a shift in heme iron spin-state equilibrium in wild-type.

### 3.2.15.2 Ionic strength effects on A264E spectral properties

In view of the proposed interactions between the anionic glutamate side chain in the A264E mutant and the heme iron, the influences of ionic strength on the visible spectral properties of the A264E mutant were investigated to assess whether changes in the ligation state could be induced by elevation of the salt (KCl) concentration of the medium.
The effects of KCl concentration on the spectral properties of A264E heme domain

The spectrum shown in red was obtained with no KCl present using 25 mM MOPS as buffer at pH 7.4, with its Soret maximum at 418 nm. The spectrum shown in blue was recorded in the same buffer with 1 M KCl and has its Soret maximum at 424 nm. Both spectra were collected using ~ 4 μM A264E heme domain.

The data presented in Figure 3.37 show clearly that it is possible to increase the proportion of glutamate ligating to the heme iron in the A264E enzyme by increasing the ionic strength of the enzyme's environment. It is difficult to predict exactly the reason for the ionic strength-induced increase in glutamate coordination in A264E. However, it is possible that increased ionic strength leads to changes in the organisation of water molecules in the active site of the enzyme, resulting in loss of the axial aqua ligand and allowing the glutamate to ligate to the iron instead. Alternatively, changes in ionic strength might affect active site molecular interaction(s) of E264 other than that with the iron, like the location(s) for the non-heme iron bound forms of E264, so promoting its ligation to the iron. Structural data, shown below, have revealed a second major position for the E264 side chain in addition to the heme iron-ligated conformation.
3.2.15.3 \textit{pH effects on A264E spectral and kinetic properties}

The effect of pH on the A264E BM3 heme domain was also studied to see if pH changes would affect the ligation state of the heme. The pH of the glutamate environment can potentially affect the charge carried by its side chain, and hence its tendency to interact with the heme iron. The pK$_a$ of free glutamic acid is 4.07, suggesting that the side chain may be predominantly negatively charged (deprotonated) in the pH range compatible with maintaining BM3 in a stable state in solution (pH 5 - 9) (223). However, if the glutamate side chain was located in a peculiar environment (e.g. the hydrophobic BM3 active site) or interacting with other moieties, then the pK$_a$ of the glutamate could be altered significantly. To examine E264 coordination to the heme iron, UV-visible spectra were collected for the substrate-free A264E heme domain in buffered solutions across a broad pH range.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.38.png}
\caption{Effect of pH on the UV-visible spectrum of A264E heme domain.}
\end{figure}

The red spectrum was measured at pH 5, the black at pH 7 and the blue at pH 9, using substrate-free A264E at a concentration of ~ 4.3 \textmu M, with the Soret bands located at 418 nm, 419 nm and 424 nm respectively. Shown in inset is the maximal absorbance change plotted against pH, which has been fitted to a sigmoidal function allowing tentative assignment of the pK$_a$ as pH 7.2 ± 0.2.

At the lowest pH tested (pH 5) the substrate-free A264E Soret band was observed at 418 nm, which is the same wavelength as that for the Soret band in the low-spin, ferric
wild-type enzyme. This suggests that a more acidic environment might retain the glutamate in the protonated glutamic acid form, preventing its ligation to the heme iron. When the pH is raised to pH 7, the Soret peak is observed at 419 nm (as is seen in the previously reported studies in assay buffer). When the pH is raised to 9, the Soret peak moves to 423 nm. The change to the longer wavelength in the basic environment probably correlates with a higher proportion of the glutamate forming a ligand to the heme iron. A plot of the change in Soret band maximum against the pH, shown in inset to Figure 3.38 describes a sigmoid and fitting of these data to the Henderson-Hasselbach function indicates a pKₐ value of 7.2 ± 0.2. This is tentatively assigned to E264. Thus, these data are consistent with the hypothesis that the glutamate has a pKₐ value increased due to its protein environment. 

Following discovery that the proportion of ligated glutamate to iron could be affected by pH, steady-state kinetics were repeated at pH 6.5 and 8.5 with the substrate arachidonate. At pH 8.5 no substrate-dependent NADPH oxidation was observed, while at pH 6.5 the catalytic parameters based on arachidonate-dependent NADPH oxidation were $k_{cat} = 948 ± 88 \text{ min}^{-1}$ and $K_M = 0.27 ± 0.08 \text{ pM}$. At pH 7.4, as was discussed in section 3.2.11, the respective $k_{cat}$ value was $1709 ± 119 \text{ min}^{-1}$. Although it might be expected that inhibiting the ligation between the glutamate and the iron would increase the rate of substrate-dependent NADPH oxidation, it also must be taken into account that earlier studies have shown that a buffer pH of ~7.4 is the optimal pH for enzyme turnover (221). Thus, a more acidic buffer pH of 6.5 may have effects on a number of factors including NADPH binding, substrate association, domain interactions that are detrimental to the catalytic efficiency of the enzyme and that might overcome any improvements that might be caused by partial inhibition of heme iron ligation by E264. It should also be recalled that, despite apparent substrate-induced coordination of heme iron in A264E, the enzyme retains considerable activity in steady-state turnover with long chain fatty acids. Thus, conclusions drawn from static optical titrations should be viewed with caution, since steady-state studies suggest that E264 can be readily displaced as an inhibitory ligand (in favour of oxygen) in enzyme turnover conditions. It is, however, expected that the steady-state turnover rate would be severely affected in a more basic environment. As was seen in Figure 3.38 the proportion of E264 heme-ligated increases with pH. Thus, there would
be an increased tendency for inhibitory heme iron ligation at high pH in addition to other detrimental effects on catalytic efficiency caused by basic buffer conditions.

3.2.16 X-ray crystallography

Protein crystallisation occurs when, under conditions of supersaturation, the protein is driven towards an equilibrium state, in which protein is partitioned between a solid and soluble phase. In passing into the solid phase the protein will move to minimise the free energy of the system by maximising attractive interactions, and minimising repulsive interactions. This energy minimisation will take two forms, the molecules may aggregate as an amorphous precipitate, or form ordered crystals. If the process of energy minimisation proceeds too quickly, precipitate will generally form. Crystallogenesis therefore aims to provide the protein with an environment which allows energy minimisation to occur through forming ordered crystals (224).

Elucidation of the structure of the A264E BM3 mutant heme domain, in both the substrate-free and substrate-bound forms provides one of the best ways to establish the nature of the novel ligation state. For this reason, crystallisation condition for the A264E heme domain in both substrate-free and palmitoleic acid-bound forms were sought.

A broad screen of various salts and other precipitants was undertaken with 100 mM cacodylic acid at pH 6.3 as buffer. The screen was employed with both substrate-free and ~35 μM palmitoleic acid-bound A264E heme domain. This concentration corresponds to approximately 6 times its $K_d$. In the substrate-free form, crystals formed in a number of the wells in which divalent cations were present. In the presence of manganese sulphate, “clumped” crystals were formed, the best of which were with the precipitant PEG 2000MME (polyethyleneglycol 2000 monomethylether). The conditions were then refined to 100 mM cacodylic acid at pH 6.3, 140 mM MnSO$_4$, 16% PEG 2000MME at 4°C. One of the crystals obtained under these conditions is shown in Figure 3.39.
Palmitoleic acid-bound crystals of A264E heme domain were optimised to 100 mM magnesium acetate, 20 % PEG 2000MME, 100 mM cacodylic acid at pH 6.3.

Crystal diffraction and structural refinement was carried out by Mr. Gordon Joyce and Dr David Leys at the ESRF (European synchrotron radiation facility) in Grenoble. Substrate-free crystals diffracted to 2 Å in the space group P2₁2₁2₁ and the substrate-bound crystals diffracted to 2.75 Å in the space group C22₂₁.

The substrate-free structure was found to have two molecules in the asymmetric unit, one of which has the E264 side chain ligated to the iron, and the other of which has the axial water molecule present.
Chapter 3 Characterisation of the A264E mutant of P450 BM3

Figure 3.40 Conformation of A264E in molecules A and B of substrate-free A264E heme domain structure
The heme is shown in red, F87, C400 (the cysteine forming the proximal heme-ligand) T260 and E264 can also be seen and are labelled in panel A. All residues and heme are shown in stick representation. Panel A shows the ligated form of the enzyme and B the unligated form where the distal water molecule can be seen above the heme iron.

Examination of the structures has suggested that the reason the ligand forms to the iron rather than interacting with the heme periphery and bonding with the heme 5-methyl group may be steric hindrance from threonine 260 or phenylalanine 87. If the ligated and unligated forms of the structure are compared, it can be seen that in the unligated form the glutamate side chain interacts with the side chain of phenylalanine 87. Potentially, further mutagenesis could be used to remove the obstructive side chains of F87 and/or T260, in this way enabling access of the E264 side chain to the heme methyl.

The palmitoleate-bound crystal was found to contain 4 molecules in the asymmetric unit, in the same arrangement as seen in the wild-type palmitoleate-bound structure (135), with the exception that all molecules showed the E264 side chain ligating to the heme iron. The structure of the heme and its surrounding residues is shown below in Figure 3.41 and discussed thereafter.
Figure 3.41 **Structure of the heme environment of palmitoleate-bound A264E heme domain**

Residues and the heme are represented stick form. The palmitoleate molecule can be seen coloured purple stacking against F87 and the A264E side chain ligating to the heme iron, with C400 below the heme ligating to the proximal position and T260 behind E264 and F87.

Figure 3.41 shows the palmitoleate molecule stacking against the phenyl side chain of F87, as is seen in the palmitoleate-bound wild-type structure (135). In molecule B of the substrate-free A264E structure, shown in Figure 3.40, the E264 residue occupies this position. The structural data show that in presence of substrate the E264 side chain is preferentially displaced from its interaction site with the F87 side chain, forcing it predominantly into its other major favoured conformation, *i.e.* with the side chain carboxylated coordinated to the heme iron. These structural data explain the substrate-induced ligation seen in spectroscopic analysis using longer chain fatty acid substrates.

Comparisons between the overall fold of the substrate-free A264E BM3 structure and that of other previously solved wild-type BM3 structures shows that the A264E mutant occupies the conformation found in the substrate-bound wild-type enzyme in both molecules of the asymmetric unit. Figure 3.42 shows a comparison between the
substrate-free A264E structure and the substrate-free wild-type structure (142), and Figure 3.43 shows a comparison between the substrate-free A264E structure and the N-palmitoylglycine-bound wild-type structure (147).

**Figure 3.42 Overlay of substrate-free A264E structure and wild-type substrate-free structure**

An α-carbon trace representation is shown with the structure of substrate-free A264E in blue, and with the substrate-free wild-type BM3 structure (PDB code 2HPD, (142), shown in green. The A264E heme is shown in ball and stick representation. For clarity the wild-type heme is not shown. Comparison of the 2 structures shows a number of conformational differences. There are substantial changes in the substrate-binding channel. The I-helix, seen across the centre of the diagram, is more kinked in the A264E mutant, and there has been substantial motion in the F and G-helices and the loop connecting them.
Figure 3.43 Overlay of structures for substrate-free A264E heme domain and N-palmitoylglycine-bound wild-type P450 BM3.

An α-carbon trace of substrate-free A264E is shown in blue, with the N-palmitoylglycine-bound wild-type structure (147) shown in red. The heme of the A264E structure is shown in ball and stick representation. For clarity, the heme of the wild-type structure is not shown. By comparing these two structures, it can be seen that the mutant and substrate-bound enzyme have essentially the same overall fold, showing that the A264E heme domain has adopted the substrate-bound conformation, even though there is no substrate present in this A264E crystal form.

Comparing the α-carbon overlays between the substrate-free A264E mutant and substrate-free/N-palmitoylglycine-bound wild-type P450 BM3 heme domains shows that the A264E mutant is no longer in the conformation seen in the substrate-free wild-type enzyme, and is instead in the same conformation as is seen in the substrate-bound wild-type structure. The presence of the larger, charged glutamate at the 264 position normally occupied by the smaller, uncharged alanine appears to have the effect of altering the conformational equilibrium of the P450 in favour of the form that has previously been observed crystallographically only in the presence of fatty acid substrate. This finding has important ramifications for our understanding of the
conformational dynamics of the P450 and its relevance to substrate binding, and is discussed in more detail below.
3.3 Discussion

The work carried out on the A264E BM3 mutant has shown successful generation of the mutation in both the heme domain and intact flavocytochrome P450 BM3. The enzyme has been spectroscopically and structurally characterised using a variety of techniques. The original aim of the work was to mutate the alanine to glutamate, mimicking the family 4 P450s and potentially generating a covalent ligation between the glutamate side chain and the heme 5-methyl group, as is seen in several members of the CYP4 family (193,194). However, the mutation did not result in any significant amount of covalent ligation of the glutamate 264 to the heme. Instead, the A264E variant exhibited glutamate ligation directly to the heme iron. In the substrate-free form, the A264E enzyme has been shown to exist in an equilibrium between the glutamate-ligated form and the water-ligated form. Structural studies show that, in the water-ligated form, the glutamate side chain interacts with the phenyl side chain of phenylalanine 87, which lies at the end of the substrate-binding channel, close to the heme, and has been shown to be important for interactions with the ω-methyl group of fatty acids substrates (171,181). Crystallographic data have also shown that there is steric hindrance to the formation of a covalent ligation to the heme 5-methyl group from the side chains of both phenylalanine 87 and threonine 260. The latter is one of the I-helix residues, the same structural element that contains E264. It is therefore possible that, by removing this steric hindrance through introduction of secondary mutations at these positions, ligation between the glutamate and heme macrocycle may be favoured, rather than the glutamate binding directly to the iron.

Spectroscopic and structural studies demonstrate that the addition of the longer chain fatty acid substrates arachidonate, palmitoleate and palmitate induce the glutamate to become almost fully ligated to the iron. From the crystallographic data it can be seen that this is because there is not space for both the glutamate and substrate to occupy space above F87 in the active site. Interaction of the ω-end of the fatty acid chain with F87 phenyl group displaces the glutamate side chain and forces it to ligate instead to the heme iron. The shorter chain fatty acids laurate and myristate do not cause ligation of the glutamate to iron to the same extent. This may be partially due to their lower occupancy (higher $K_d$ values), but is also likely due to their shorter chain length, which (together with fatty acid mobility in the active site) probably renders them less
effective at displacing the glutamate side chain from its interaction position with F87. The A264E flavocytochrome enzyme is still able to catalyse turnover of all the substrates tested, as was shown by both steady-state kinetics and product isolation. This is likely due in part to a small amount of non glutamate-coordinated enzyme remaining at all times. However, since the longer chain fatty acids induce almost complete coordination of the heme iron by the glutamate side chain, it is more likely that the high rates of catalytic turnover (and coupling) seen with these substrates results from displacement of the glutamate ligand during redox cycling of the enzyme, enabling access of molecular oxygen to the heme iron and facilitating reductive scission of bond dioxygen and oxygenation of substrates, as seen for the wild-type enzyme. Clearly turnover-dependent displacement of the glutamate ligand is an interesting phenomenon which merits further study.

The second major discovery from this work on the A264E variant is that, in the substrate-free form, the enzyme displays the conformation of the substrate-bound wild-type enzyme. A number of structures have been solved for wild-type P450 BM3 heme domain in both the substrate-free form (117,142) and the substrate-bound form (135,147). In each of these structures, the same major differences are seen between the substrate-free and substrate-bound structures. These changes are discussed in section 1.5 of the Introduction. The structure of the substrate-free A264E heme domain shows two forms in the asymmetric unit, one of which has an axial water molecule on the iron, the other of which has the glutamate side chain ligated to the iron. Both forms are in the previously supposed “substrate-bound” (SB) conformation, regardless of the absence of substrate or the nature of the 6th axial ligand to the iron (water or glutamate). The presence of the axial water, while the enzyme is in the SB conformation, contradicts, in part, some work carried out by Haines et al (147). They showed, using structural studies of the N-palmitoylglycine-bound wild-type heme domain, that the expected SB conformation was observed, and a water molecule was situated in the environment of the heme, but to the side against the I-helix resting beside threonine 268. They suggested that this water was the same molecule that occupied the distal position on the heme iron, and the movement of the water from the axial ligand position towards the I-helix by 1 Å is a major factor responsible for the conformational changes observed between the substrate-free (SF) and SB conformations. Further, they suggested that novel hydrogen bonding interactions with
this water molecule drive the conformational change to the SB form. The structure of A264E heme domain in the absence of substrate shows the water molecule in the axial position in one of the two molecules. In the other unit (where the glutamate coordinates the iron), the water is displaced but does not occupy the secondary position observed by Haines et al. (147). This is apparently contradictory to their suggestion that the movement of the water is responsible for the change in conformation.

The data obtained from structural and spectroscopic analysis of the various A264E forms lead to a simpler explanation for the conformational changes observed in the BM3 enzyme. The fatty acids tested in optical binding titrations were found generally to bind to the A264E mutant tighter than to the wild-type enzyme. This in turn, suggests that the fatty acids bind more tightly to BM3 in its SB conformation than in its SF conformation. This suggests that, rather than substrate binding causing displacement of the distal water molecule from the heme iron and a subsequent conformational rearrangement, the enzyme exists instead in a natural equilibrium between the SF and SB conformations in the absence of substrate, with the distal water molecule present in both cases. It therefore follows that substrate binds preferentially to the SB conformation, displacing the distal water molecule but not otherwise causing significant further structural rearrangement. Instead, fatty acid binding should stabilize the SB conformation. This leads to the hypothesis that the wild-type enzyme in its fatty acid free form exists in a conformational equilibrium between the SF and SB forms, with the SF form predominating. The SB form has higher affinity for fatty acids, and these bind favourably to this form with the effect that the equilibrium is pulled over towards the SB conformation. In the A264E enzyme, the equilibrium (as a result of the I helix mutation and regardless of whether the E264 side chain interacts with heme iron or F87) lies in favour of the SB conformation. As a result, substrate binding is tighter to this enzyme. This therefore suggests that previous assumptions in the field that substrate binding induces conformational change in P450 BM3 are incorrect. Instead it appears that the conformational equilibrium is a natural one that can be perturbed by active site mutation, and that substrates bind favourably to one (the SB) of the two major conformations populated. Further, the heme iron redox potential of the A264E mutant enzyme was shown to be unaffected by the presence of substrate, and there is no significant spin-state shift (towards high-spin) observed on substrate binding to the enzyme. However, the redox potential of the heme iron is higher for the
Chapter 3 Characterisation of the A264E mutant of P450 BM3

A264E protein than for the wild-type enzyme, suggesting that the conformation of the enzyme also has some bearing on the redox potential of the heme iron in the P450 BM3 system.

In conclusion, this work has therefore challenged a number of previously accepted hypotheses concerning the structure of P450 BM3 and its structural rearrangement in the presence of substrate(s). This work has also provided spectroscopic fingerprint data for use in the analysis of novel cytochrome molecules with unusual heme iron ligation states. It is evident that positioning of other amino acids at residue 264 might result in further, novel heme iron ligation states, and this is the subject of a following chapter in this thesis. The data presented in this chapter have been published as two papers, which are included in appendices following all the results chapters (148, 225).
Chapter 4 Characterisation of A264K/Q/H mutant forms of flavocytochrome P450 BM3 and its heme domain: Novel P450 heme ligation states
4.1 **Introduction**

In the preceding chapter I have investigated the properties of the cytochrome P450 BM3 A264E mutant. The A264 residue is positioned in the I-helix where it is in close proximity to the distal face of the heme iron. It was shown that under certain conditions the mutant E264 residue ligates to the distal position of the heme iron. As a continuation to this work a further series of mutations have been made to the A264 residue, with the aim of generating a series of novel heme iron ligation states involving the side chains of the new amino acids. This chapter discusses the generation and subsequent characterization of mutants of alanine 264 to the nitrogen-containing amino acids lysine, histidine and glutamine (*i.e.* A264/K/H and Q).

There are a large number of cytochromes with their heme iron ligated to the nitrogen group of amino acids, but to date very few have been discovered with heme ligated in both the distal and proximal position and in which one of the ligands is cysteine.

The photosynthetic bacterium *Rhodospirillum rubrum* is capable of utilising carbon monoxide as its sole energy source (226). The carbon monoxide sensing molecule CooA binds CO, then activating expression of two operons, the gene products from which are capable of oxidising CO and therefore using it as a sole energy source (227). The CO sensor, CooA is a dimeric heme *b* containing cytochrome, which in the ferrous CO-free form has histidine and proline axial heme ligations, with the N-terminal proline originating from the polypeptide chain of the first molecule in the dimer as the axial ligand to the heme in the second molecule, and *vice versa* (228). In the ferrous state the cysteine ligation is lost, and replaced by a histidine, giving His/Pro ligation (229), and the proline is then displaced by CO, allowing DNA to bind and the expression of CO oxidising gene products (230). The ligand switches are rare, and to date no other protein with proline-heme ligation has been discovered.
Figure 4.1  **Ferrous CO-free CooA heme and ligands**
The heme group is shown in red, with the iron as a ball. Shown ligating to the heme iron on the lower face is His 77, with Pro 2 ligating at the upper face. Also shown is Cys 75, which ligates to the heme in place of His 77 in the ferric state. The diagram was generated from the structure solved by Lanzilotta *et al.* (228), PDB code 1FT9, using Pymol.

A second example of a non-P450 cytochrome with cysteine axial ligation is SoxAX. SoxAX is one component of the thiosulfate-oxidising multi-enzyme system mechanism (TOMES) in the photosynthetic purple bacterium *Rhodovulum sulfidophilum* (231). The TOMES mechanism is responsible for the oxidation of thiosulfate, with the derived electrons used in photosynthetic or respiratory electron transport chains (232). As yet, no role has been discovered for SoxAX, although a number have been suggested including that it may act as a specific electron mediator or be responsible for oxidation of substrate to allow it to bind to the SoxY component of the pathway (232). It is a heterodimeric protein composed of SoxA and SoxX subunits. SoxA contains two c-type hemes both with cysteine/histidine coordination and SoxX contains one c-type heme with methionine/histidine coordination (233). Interestingly heme 2 of the SoxA subunit is thiolate ligated, but the cysteine residue has been modified, post-translationally, to cysteine persulfide. It is thought that the cysteine is modified in enzyme turnover and stabilised by its protein environment and ligation to the heme group (234).
The third non-P450 cytochrome known to contain cysteine as a heme axial ligand is human cystathionine $\beta$-synthase. Cystathionine $\beta$-synthase catalyses the condensation of serine and homocysteine resulting in formation of cystathionine, as the first step of a pathway which ultimately results in cysteine formation (235). Deficiency in cystathionine $\beta$-synthase results in homocystinuria, which is an inherited disease that can result in skeletal problems, vascular disease and mental retardation (236).

Cystathionine $\beta$-synthase is a homotetramer, with each subunit having one $b$-type heme and one pyridoxal 5'-phosphate (235,237). The heme group has cysteine and histidine axial ligands (238), with the cysteine residue in the deprotonated thiol form (239). It is thought that the heme groups are not required for catalytic activity (240), and the yeast cystathionine $\beta$-synthase does not contain any heme (241), but it has also been shown that the redox state of the heme regulates enzyme activity. When the heme is in the ferrous state the enzyme activity is found to be 1.7 fold slower, returning to full activity when the heme is oxidised. It has been postulated that this redox sensitivity may mediate the enzyme activity in respect of the redox status of the cell, regulating homocysteine's passage through the pathway which ultimately leads to sulfate or methionine (242).
Figure 4.3 Cystathionine β-synthase heme structure
Shown in red is the heme group of one monomer, with the iron shown in spacefill representation. The ligating cysteine (C52) and histidine (H65) residues can be seen above and below the heme iron. The figure was generated from the structure of Meier et al. using Pymol (237). PBD code 1JBQ.

The three examples discussed above are, with one notable exception, the only non-P450 cytochromes with cysteine axial ligation which have been discovered to date. The other example is the family of nitric oxide synthase enzymes (NOS) which were discussed in Chapter 1. They catalyse the two step formation of nitric oxide and L-citrulline from L-arginine in a process which utilises 2 electrons and molecular oxygen. Like the cytochromes P450 the NOS enzymes have cysteine thiolate ligation to their heme, and water as distal ligand, but differ in overall fold.

In view of the fact that relatively few types of heme iron coordination involving cysteine/cysteinate have been recognized in nature, it is of interest to exploit the BM3 enzyme to determine whether further substitutions at A264 can produce further novel types of heme iron ligand sets of the form Cys-Fe-X where X is a side chain donated by the amino acid substituted for A264 by mutagenesis. In this chapter, the amino acids introduced at position 264 have nitrogen-containing side chains and the aim is to produce variants in which the aqua distal ligand to the heme iron is replaced by one or more of these side chains (K/H/Q). The chapter reports the generation of the mutants at the DNA level, the expression and production of the A264K/H/Q variant proteins and the characterization of these proteins with respect to their catalytic activity, heme iron ligation state and spectroscopic properties, and atomic structures. This chapter
reports on the characterisation of further novel heme iron ligand sets for the A264K/H/Q mutants in both the heme domain and intact flavocytochrome forms.
4.2 Results

4.2.1 Mutagenesis

The A264K, A264H and A264Q mutants were all successfully generated in both the intact flavocytochrome and heme domain of P450 BM3, in the plasmids pBM25 and pBM20, respectively, as described in the Methods. Following the mutagenic PCR reactions, the reaction mixtures were digested with the restriction endonuclease DpnI, to remove non-mutated template DNA. 5 μl of the total PCR reaction mixtures were then run on a 0.8 % agarose gel to verify the success of the mutagenic PCR reaction. The agarose gel run to verify the A264K heme domain mutagenesis is shown in Figure 4.4.

![Ethidium bromide-stained 0.8 % agarose gel of DpnI digested A264K heme domain PCR product](image)

Figure 4.4 Ethidium bromide-stained 0.8 % agarose gel of DpnI digested A264K heme domain PCR product
Lane 1 shows 5 μl of A264K heme domain PCR product and lane 2 NEB 1 Kb DNA marker. The 3 Kb marker band contains 50 ng DNA, comparison with lane 1 suggests there is approximately 80 ng DNA in lane 1 at a size of ~ 5 Kb. The presence of DNA of the correct size (4584 bp) indicates the successful PCR.

Following transformation into Novablue™ competent cells and growth on agar plates containing ampicillin, 6 colonies were picked for each mutant and plasmids prepared from derived cultures, as described in the Methods section. The A264K and A264Q mutagenic primers contained silent mutations which gave an additional BspHI restriction enzyme site, digestion with BspHI therefore verified the presence of the mutation, digested A264K heme domain is shown in Figure 4.5.
Chapter 4 Characterisation of A264K/H/Q P450 BM3 mutants

Figure 4.5 Ethidium bromide-stained 0.8 % agarose gel of BspHI digested A264K heme domain plasmid constructs.
The gel shows bands corresponding to DNA fragments of 1895 bp, 1150 bp, 1008 bp, 426 bp and 105 bp, which are the calculated DNA fragment sizes of pBM20 digestion with BspHI following successful mutagenesis to generate the A264K mutant. 4 samples are shown, from 4 separate minipreps. A sample (~50 ng) of NEB 1 Kb marker is shown in the right hand lane with the size of relevant marker bands indicated.

To verify that the mutations were successfully introduced and to check that no secondary mutations had been introduced, the heme domain BM3 gene, or flavocytochrome BM3 gene was sequenced by the dideoxy chain termination method using primers detailed in Table 2.11 of Chapter 2 (PNACL facility University of Leicester). A chromatogram of the sequencing data for the A264K sequence is shown below in Figure 4.6.

Figure 4.6 A264K heme domain chromatogram
The oligonucleotide primer used to generate the mutant is underlined and the 264 lysine codon boxed in red. The entire sequence corresponds to the wild-type sequence, with the exception of the mutations intentionally introduced by the use of the primer in mutagenic PCR.
Chapter 4 Characterisation of A264K/H/Q P450 BM3 mutants

The A264H mutagenic primer ultimately used did not contain silent mutations to generate an additional BspHI restriction endonuclease site. Initially, a primer was designed containing the BspHI site, but the PCR reaction was repeatedly unsuccessful, so primers were redesigned with fewer mismatches in order to increase the probability of the primers annealing to the backbone DNA. The successful PCR was verified by DNA sequencing of heme domain and full length flavocytochrome P450 BM3 A264H clones using the same primers as those used to verify the A264K and A264Q mutants.

4.2.2 Overexpression and purification of mutant BM3 enzymes

Mutant and wild-type enzymes, in both heme domain and intact flavocytochrome constructs, were successfully purified as described in the Methods. Figure 4.7 shows a 10 % SDS-PAGE gel of purified A264K heme domain. All other mutants and wild-type proteins were also purified to the same standard of purity and verified by SDS-PAGE gel analysis. No significant alterations in protein stability were noted as a result of mutations (i.e. no significant increases in proteolysis of the mutants with respect to wild-type P450 BM3 were observed).

![Figure 4.7 Pure A264K heme domain](image)

A 10% SDS-PAGE gel is shown, with purified A264K heme domain in lane 1 and NEB pre-stained broad-range marker in lane 2, with band sizes indicated. Comparison with the marker protein shows that the A264K mutant is the expected size (54 KDa).
Chapter 4 Characterisation of A264K/H/Q P450 BM3 mutants

4.2.3 Calculation of heme concentration

The concentration of the mutants was calculated by generating pyridine hemochromagen spectra by the method of Berry and Trumpower (190). Generally, the method of Omura and Sato (31) has been used to calculate the concentration of cytochromes P450, but the A264K and A264H mutants showed no conversion of the Soret maximum to the 450 nm form following incubation with the reductant sodium dithionite and carbon monoxide, and the A264Q mutant showed incomplete conversion to 450 nm. As will be discussed later in the chapter, the reason for failure of the A264K and A264H mutants to form carbon monoxy adducts is the relative stability of the Cys/Lys- and Cys/His-coordinated heme iron complexes in the mutants.

![Pyridine hemochromagen spectra of A264K, A264H and A264Q heme domains](image.png)

**Figure 4.8** Pyridine hemochromagen spectra of A264K, A264H and A264Q heme domains

Shown in blue the A264K mutant, in red A264H and in green the A264Q mutant. Each of the mutants and the wild-type enzyme have maxima in the Q-band (i.e. alpha/beta band region) at 556 nm, which is typical of heme b. Using the extinction coefficient of 24 mM⁻¹cm⁻¹ for A550 nm – A535 nm the concentrations of each sample shown were calculated as 6.7 μM, 5.6 μM and 6.15 μM respectively.
4.2.4 Comparison of wild-type and A264Q/K/H mutant UV-visible spectra

Wild-type and A264Q/K/H mutant heme domain UV-visible spectra in their oxidised (ferric), substrate-free forms are shown below in Figure 4.9.

![Absorbance spectra of oxidised, substrate-free wild-type, A264Q, A264K and A264H heme domains of P450 BM3](image)

Figure 4.9 Absorbance spectra of oxidised, substrate-free wild-type, A264Q, A264K and A264H heme domains of P450 BM3

All spectra were collected using ~ 8 μM BM3 heme domain. Wild-type, shown in black, has a Soret maximum at 418 nm. A264Q (green) also has a Soret maximum at 418 nm. A264K (blue) has a Soret maximum at 424 nm, and A264H (red) has a Soret maximum at 427 nm.

<table>
<thead>
<tr>
<th>Table 4.1</th>
<th>UV-visible spectral maxima for wild-type and A264H/K/Q mutants</th>
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<tbody>
<tr>
<td></td>
<td>Soret (nm)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>418</td>
</tr>
<tr>
<td>Wild-type + Substrate</td>
<td>390</td>
</tr>
<tr>
<td>Wild-type + 4 Phelm</td>
<td>424</td>
</tr>
<tr>
<td>A264H</td>
<td>427</td>
</tr>
<tr>
<td>A264K</td>
<td>424</td>
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<td>A264Q</td>
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Comparison of the heme domain spectra for the oxidised A264Q/K/H mutants and the wild-type reveals significant changes between the mutants and wild-type enzyme in their oxidised forms. The wild-type spectrum is that of a typical, ferric low-spin P450 with cysteinate-water coordination of the heme iron. The similarity of the spectrum for the A264Q mutant suggests that heme iron coordination may not be significantly perturbed from wild-type. The A264H and A264K mutants, however, show a shift in their Soret maxima to 424 nm and 427 nm, respectively. This shift is likely to reflect ligation of the H264 and K264 amino acid side chains to the iron at the distal position via basic nitrogen groups. Based on preceding studies of the A264E mutant (Chapter 3), it is expected that these mutant P450s retain cysteine thiolate ligation to heme iron at the proximal position. As a direct comparison of the A264H mutant with another imidazole/cysteinate iron complex of P450 BM3. The spectrum of wild-type P450 BM3 heme domain bound to the inhibitor 4-phenylimidazole is shown in Figure 4.10.

Figure 4.10 4-phenylimidazole binding to wild-type P450 BM3
The red spectrum is that of ~5.5 μM wild-type heme domain and the blue spectrum is that recorded following the addition of 4-phenylimidazole to a final concentration of 32 μM. In the absence of 4-phenylimidazole the Soret maximum is seen at 418 nm with α-band at 569 nm and β-band at 535 nm, and in the presence of 4-phenylimidazole at the Soret, α-band and β-band are found at 424 nm, 574 nm and 541 nm respectively.
Chapter 4 Characterisation of A264K/H/Q P450 BM3 mutants

Comparison between the 4-phenylimidazole bound wild-type spectrum (shown in Figure 4.10) and the spectra of the substrate-free oxidised A264K and A264H mutants (Figure 4.9) shows that the ligand-free forms of the mutants share a number of spectral properties with the 4-phenylimidazole-bound wild-type enzyme. Both ligand-free A264H and A264K show a shift in their Soret maxima to longer wavelength, as observed for 4-phenylimidazole-bound wild-type heme domain. There are also similarities in the spectral changes seen in the region of the \( \alpha \) and \( \beta \) bands, with the \( \alpha \) band losing intensity, the \( \beta \) band increasing in intensity and both shifting to longer wavelength by \( \sim 6 \text{nm} \). In the case of the A264H mutant, the fact that the Soret wavelength maximum is located at exactly the same point as that for the 4-phenylimidazole-ligated wild-type enzyme indicates strongly that both are distally coordinated by an imidazole group (that of His264 in the case of A264H). In the case of A264K, the further red shift of the Soret maximum in A264K suggests strongly that the lysine side chain is distally coordinated to the heme.

The spectrum of the A264Q mutant does not show any considerable differences in the position of the Soret \( \alpha \)- or \( \beta \)-band with respect to that seen in the wild-type enzyme. Therefore no differences between heme iron coordination in the A264Q mutant and wild-type enzyme can be inferred at this stage.

4.2.5 Substrate-binding

The substrates arachidonic acid, palmitoleic acid, palmitic acid, myristic acid and lauric acid were titrated against the wild-type, A264K, A264H and A264Q BM3 heme domains. As has been reported previously (e.g. (76, 106)), and shown in Chapter 3, the wild-type enzyme shows a shift in the Soret maximum as the enzyme goes from a low-spin ferric form to a high-spin ferric form upon addition of substrate, changing from 418 nm to 390 nm. The enzyme is apparently incapable of shifting to a 100% high-spin form within the substrate solubility ranges in aqueous buffers, and shows varying degrees of spin-state conversion with different substrates, according to their relative affinities and solubilities. The largest shift to the high-spin form is seen with the 20 carbon, polyunsaturated fatty acid arachidonic acid. The spectral shift seen using arachidonic acid with the wild-type enzyme is shown in Figure 4.11.
Figure 4.11  **Arachidonate binding to wild-type P450 BM3 heme domain**

The blue spectrum shows ~5 μM low-spin wild-type enzyme and the red spectrum was recorded following the addition of arachidonic acid to a final concentration of 7 μM. Shown in the inset is the plot of maximal absorbance change observed (peak minus trough from difference spectra) against the corresponding arachidonate concentration. Data have been fitted to the quadratic tight-binding equation (Equation 2), with the enzyme concentration fixed as 5.2 μM. This gave an A_max of 0.14 ± 0.002 and an apparent K_a of 0.82 ± 0.05 μM.

The addition of any of the fatty acid substrates listed above to the A264K or A264H heme domains failed to induce any significant optical changes. A typical example is shown in Figure 4.12.
Chapter 4 Characterisation of A264K/H/Q P450 BM3 mutants

Figure 4.12  A264H substrate-binding titration

The red spectrum shows ~5.5 μM A264H heme domain and the blue spectrum was recorded following the addition of arachidonic acid to a final concentration of 33 μM. At this concentration, there is induction of a near-complete conversion to the high-spin form in the wild-type enzyme. Both the A264H mutant spectra shown have their Soret maxima at 427 nm.

The addition of a range of fatty acid substrates to the A264K and A264H mutants failed to induce any significant spectral shift. These findings are consistent with the observations made from analysis of the ferric mutant spectra, which suggested that nitrogenous ligation of the heme iron from the H264 and K264 side chains occurs to near completion in the mutants. In the wild-type P450 BM3, fatty acid binding results in displacement of the weakly-bound water molecule from the distal coordination position. In A264K and A264H, substrate appears to be unable to displace the ligand, either because the coordinating nitrogen from the K264 or H264 side chains is a substantially stronger ligand than is the water in wild-type P450 BM3, or (as was shown in Chapter 3 with the A264E mutant) the substrate access channel is blocked by the mutant residue.
Figure 4.13  Binding of palmitoleate to A264Q BM3 mutant heme domain

Palmitoleate was titrated as 0.1 μl aliquots from a 19.5 mM stock solution against ~4.8 μM A264Q heme domain. Shown in red is the starting spectrum prior to the addition of any palmitoleate. Shown in blue is the final spectrum collected containing palmitoleate at the near-saturating concentration of 21.45 μM. The spectra shown in black were collected at intermediate palmitoleate concentrations. Shown in inset are data points generated from the plot of maximal absorbance change (derived from peak minus trough data in difference spectra) against the corresponding substrate concentration. The data have been fitted to a rectangular hyperbola (Equation 2) generating a $K_d$ of 6.43 ± 0.51 μM.

Shown above in Figure 4.13 are the spectral data derived from the binding of the substrate palmitoleate to the A264Q mutant BM3 heme domain. Binding of substrate has induced a typical low- to high-spin shift in the heme iron, with the Soret maximum shifting from 418 nm towards 390 nm. However, only a small proportion of high-spin P450 is formed at substrate concentrations that are near-saturating for the wild-type enzyme and which are at the limits of solubility of the fatty acid in the reaction mixture. A far more extensive perturbation of absorption is observed for the wild-type enzyme when titrated with palmitoleate. The isosbestic point seen at 409 nm on addition of substrate indicates strongly that there is a simple conversion of low-spin
and high-spin species occurring on fatty acid addition to the mutant. Given that the apparent binding constant for palmitoleate is not considerably altered from that of the wild-type enzyme (6.43 μM versus 3.5 μM for wild-type), changes in affinity cannot explain the altered spin-state shift behaviour. However, it is possible, for example, that the enzyme exists in a heterogeneous conformational state, where one conformer is able to bind substrate and shift heme iron spin-state equilibrium from the low- to the high-spin form, and in which one or more further forms are unable to bind substrate or bind it in a mode that does not result in altered heme iron spin state. This model would be in some way consistent with the data reported for the A264E mutant in Chapter 3, where apparent tighter binding of fatty acids was correlated with alterations in conformational equilibria. In the case of the A264Q mutant, it may be the case that a lower proportion of enzyme is able to bind substrate in a manner conducive to spin-state perturbation. Spectral changes observed do not indicate whether any of the protein has a novel ligation by the Q264 side chain. If this is the case, then it would be true that the Soret maximum is near-identical to that of the aqua-ligated wild-type and that at least a proportion of the ligand can be displaced by fatty acid binding. On the basis of preceding studies with the A264E mutant, substrate-induced displacement of an amino acid ligand to the heme iron appears counter-intuitive. An alternative explanation for the small degree of palmitoleate-induce spin-state change in the A264Q mutant might be that steric effects of the new side chain prevent substrate from binding to the same extent as in wild-type. The shorter chain fatty-acids, palmitate, myristate and laurate did not give a significant A264Q spectral change within their range of solubility. Turbidity of the solution due to both substrate precipitation and protein aggregation was noted by the stage that any of these fatty acids produced any real changes in heme optical properties. This, in turn, prevented useful data collection for K_d determinations. Wild-type P450 BM3 shows a more substantial conversion to the high-spin form with the shorter chain fatty acids, enabling K_d values to be determined (see section 3.2.5). The apparent lack of spectral changes for the A264Q enzyme with shorter chain fatty acids is also in agreement with data obtained for the A264E mutant as shown in Chapter 3, where it was concluded that the shorter chain, weaker-binding fatty acids were unable to displace the glutamate side chain from its other “favoured” position against F87 and onto the heme iron. Thus, minimal spectral changes were induced. The results from preliminary optical substrate-binding assays
with A264Q suggest that the protein may be conformationally altered from wild-type P450 BM3, but do not provide categorical data as regards coordination state of the heme iron in substrate-free or substrate-free forms. The binding data presented in this section for the A264Q/K/H mutants will be discussed in more detail in relation to EPR, MCD and structural data in Sections 3.2.8 and 3.2.9 respectively.

4.2.6 Inhibitor binding

As was discussed in Chapter 3, a number of molecules act as P450 inhibitors, ligating strongly and directly to the heme iron, and thus inhibiting the enzyme by preventing substrate-induced spin-state change, heme iron reduction, oxygen binding and progression through the P450 catalytic cycle.

The inhibitors 4-phenylimidazole and sodium cyanide were titrated against wild-type and each of the A264Q/K/H heme domain mutants in order to compare relative affinities for the inhibitors and to establish (particularly in the case of A264K/H) whether the inhibitors could displace the novel distal ligands provided by the amino acid side chains. The effects of the inhibitor 4-phenylimidazole on the spectral properties of the wild-type enzyme are shown in Figure 4.10, where it can be seen that it causes a shift in the soret maximum from 418 nm to 424 nm with additional shift in the relative intensities and positions of the \( \alpha \) and \( \beta \) bands. Neither the A264K nor A264H mutants gave any spectral shift when either 4-phenylimidazole or sodium cyanide was added at concentrations that are near-saturating for the wild-type enzyme (40 \( \mu \)M and 5 mM, respectively). This is attributed to stronger nitrogenous ligation of the lysine and histidine side chains to the heme iron than that afforded by either 4-phenylimidazole or sodium cyanide. The A264Q mutant heme domain did, however, show spectral changes with both 4-phenylimidazole and sodium cyanide. The spectral changes observed are shown below in Figure 4.14 and Figure 4.15, and are discussed thereafter.
Figure 4.14 Binding of 4-phenylimidazole to A264Q P450 BM3 heme domain

0.1 μl aliquots of a 50 mM stock of 4-phenylimidazole were added to ~4.5 μM A264Q mutant heme domain. Shown in red is the starting spectrum, collected prior to the addition of any 4-phenylimidazole. Shown in blue is the final spectrum collected following the addition of a near-saturating concentration of 0.7 mM 4-phenylimidazole. Shown in black are a number of intermediate spectra collected during the titration. Difference spectra were constructed from the data collected and the maximal absorbance change derived from these data plotted against the corresponding 4-phenylimidazole concentration, shown in the inset. The data were then fitted to a rectangular hyperbola (Equation 2), giving a $K_d$ of $390 \pm 27 \mu M$. 

The apparent binding constant of 4-phenylimidazole (390 μM) is rather weaker than that for the wild-type P450 BM3 heme domain (3.8 μM), perhaps suggesting steric obstruction and/or environmental factors in the vicinity of the A264Q heme iron that weaken its binding. Alternatively, changes in conformational equilibrium of the A264Q enzyme may result in altered affinity for the substrate.

Pyridine binding results in a considerably different spectral change from that seen with the wild-type enzyme (Chapter 3 Figure 3.18), the spectrum of which shows a shift from 413 nm to 445 nm at the Soret maximum. By plotting the maximal
Chapter 4 Characterisation of A264K/H/Q P450 BM3 mutants

Figure 4.15 Sodium cyanide binding to A264Q mutant heme domain
Sodium cyanide was titrated against ~5.5 μM A264Q heme domain, as progressive 0.1 μl aliquots from a 1 M stock. Shown in red is the starting spectrum which contains no sodium cyanide, and shown in blue is the final spectrum collected, which contains near-saturating sodium cyanide at 7.2 mM. The spectra shown in black were collected at intermediate concentrations of sodium cyanide. Difference spectra were generated from the absolute optical data and the maximal absorbance change derived from these data plotted against the corresponding sodium cyanide concentration, shown inset. The data were fitted to a sigmoidal function, gave a half saturation concentration of 3.38 ± 0.02 mM.

The apparent binding constant of 4-phenylimidazole (390 μM) is rather weaker than that for the wild-type P450 BM3 heme domain (5.8 μM), perhaps suggesting steric obstruction and/or environmental factors in the vicinity of the A264Q heme iron that weaken its binding. Alternatively, changes in conformational equilibrium of the A264Q enzyme may result in altered affinity.

Sodium cyanide binding results in a considerably different spectral change from that seen with the wild type enzyme (Chapter 3 Figure 3.18), the spectrum of which shows a shift from 418 nm to 445 nm at the Soret maximum. By plotting the maximal
absorbance change induced (from peak and trough values in the difference spectrum) against the sodium cyanide concentration, the $K_d$ value for wild-type P450 BM3 was calculated as $1.68 \pm 0.68$ mM when fitted to a rectangular hyperbola. However, the cyanide binding curve for the A264Q enzyme does not describe a rectangular hyperbola, describing instead a sigmoid. The Hill function (describing cooperative binding) was fitted to the data, producing a reasonable simulation of the data and a Hill coefficient value greater than one. It is plausible that there is cooperativity in the binding of sodium cyanide to the A264Q mutant, and/or there is conformational rearrangement on binding of sodium cyanide. However, another possibility might be that the unusual binding curve represents two (or more) conformationally distinct and/or ligation-state-altered species of the A264Q protein, which bind cyanide with very different affinities. The levelling off of the absorption change versus [cyanide] plot (Figure 4.15, inset) at high cyanide concentrations suggests that the binding process has reached near-completion. The fact that a single Soret isosbestic point is seen at 430 nm also suggests that there may be only two species predominant in the reaction solution (one with cyanide ligated to the heme iron and one without) at the end of the titration. The apparent incomplete conversion of the A264Q enzyme to the cyanide-ligated form may again (as with the 4-phenylimidazole titration) reflect the presence of different conformers of the mutant that have different affinities for cyanide. Regardless of the origins of the unusual ligand binding properties of A264Q and of whether the glutamine might ligate the heme iron, it is evident that the mutation has caused some considerable disruption to the ability of the enzyme to undergo both substrate-induced spin-state change and to bind heme-coordinating inhibitors. Clearly, structural and spectroscopic data are of equal importance in characterizing the unusual behaviour of this variant as they are in analysing the A264K/H mutants.

4.2.7 Effects of ionic strength, pH and temperature

In Chapter 3 it was shown that ionic strength, temperature and pH had a significant effect on the spectral properties of the A264E P450 BM3 mutant. Substrate and inhibitor have been shown to have negligible effect on the UV-visible spectral properties of the A264K or A264H P450 BM3 mutants, but temperature, pH or ionic strength may affect them and induce changes, e.g. changes in extent of ligand
coordination and/or give rise to other spectral perturbations. For this reason, the effects of temperature, pH change and ionic strength were studied in each of the 3 mutants.

4.2.7.1 Effects of ionic strength

As was shown in Chapter 3, wild-type P450 BM3 shifts from low to high spin as the salt concentration is increased, possibly due to the high ionic strength causing displacement of the distal water molecule. The UV-visible spectra of the A264K, A264H and A264Q mutants were collected with KCl at concentrations between 0 and 3 M, in 25 mM MOPS buffer at pH 7.4. None of the mutants showed any spectral changes with varying KCl concentration. The A264K and A264H mutants have been shown above to be unaffected by the presence of substrate or inhibitor. These ionic strength data again suggest that they have formed strong K264/H264 ligations to the heme iron which are not disrupted at high ionic strength. The A264Q mutant also shows no significant spectral change in the presence of KCl, but unlike the A264K or A264H mutants has been shown to undergo spectral perturbations on binding of substrates and inhibitors. The amino acid glutamate is a charged polar amino acid and the presence of potassium ions affects interaction of the E264 side chain with the heme iron and other amino acid side chains in the active site, as was shown in Chapter 3. In contrast, ionic strength changes may have less effect on the molecular interactions of the glutamine side chain. Binding titrations carried out with the A264Q mutant suggested that a proportion of the enzyme retains water as its distal ligand. It is therefore surprising that this proportion of the enzyme is apparently unaffected by changes in ionic strength. However, it is possible that the Q264 residue is shielding the distal water from the effects of changes in ionic strength, or that a different proportion of the A264Q molecules in solution at ambient temperature have axial water ligation by comparison with those detected in the spectroscopic analyses.

4.2.7.2 Thermal effects on P450 spin state and stability

As was shown in Section 3.2.15.1 of Chapter 3, elevating temperature in the wild-type enzyme causes a shift in the heme iron spin-state equilibrium of the enzyme from low- to high-spin, almost certainly by causing displacement of the distal water molecule. The effects of temperature were studied in the A264K, A264H and A264Q mutants in
the absence of substrate or reductant. Each of the mutants was incubated at a concentration of ~10 μM for 2 minutes at 2 °C temperature increments between 10 °C and 60 °C, prior to recording UV-visible spectra. The A264K and A264H mutants showed no significant spectral shifts in the range tested, again suggesting that the mutant lysine and histidine residue side chains ligate strongly to the distal heme iron, rigidly holding the heme in the mutant enzymes in a stable hexa-coordinate conformation. The A264Q mutant was affected by temperature and the data are shown below.

Figure 4.16 Effects of temperature on A264Q heme domain
Spectra were collected for ~10 μM A264Q heme domain. Shown in red is the starting spectrum, collected at 10 °C, shown in blue is the final spectrum collected at 60 °C with spectra collected at intermediate temperatures shown in black. All spectra have their Soret maximum at 418 nm. The decrease in absorption at 418 nm with concomitant development of a species with absorption maximum at around 360 nm (at higher temperatures) likely reflects the denaturation of the P450 and the dissociation of the heme from the protein matrix.

Elevation of temperature has shown no apparent spectral changes indicative of a low- to high-spin shift in the A264Q mutant heme iron, dissimilar to the data shown for the wild-type enzyme (Figure 3.35 Chapter 3). Between 10 °C and 46 °C there is little
change in the spectrum of the A264Q mutant, with no shift towards either high-spin iron, or suggesting changes in the distal iron ligation. Between 46 °C and 60 °C the Soret maximum appears to collapse, with a concomitant increase in the absorption at ~359 nm which can probably be attributed to loss of the heme group from the polypeptide. Therefore, in the A264Q mutant temperature does not cause accumulation of the high-spin species as seen in the wild-type enzyme, but the enzyme does appear to lose its heme from the protein matrix at higher temperatures as the protein unfolds. The A264Q mutant has therefore not had the same stabilising effect as seen in the A264K and A264H mutants, but has instead destabilised the enzyme, making it more susceptible to heme loss at extremes of temperature, when compared to the wild-type enzyme.

4.2.7.3 pH effects on P450 spectral properties
The amino acids lysine and histidine both contain charged side chains, with pKₐ values of 10.54 and 9.33 respectively in free solution, with glutamine containing an uncharged, polar side chain. The UV-visible spectra of the A264K, A264H and A264Q mutants were studied at varying pH values in order to see if the ligation states of the enzyme could be altered as the charge of the 264 side chain was changed, since the pKₐ values of the K/H side chains might be shifted to values closer to neutral as a consequence of their environments within the P450 protein. UV-visible spectra were collected for each of the mutants in the pH range pH 5 to pH 9, with 100 mM potassium phosphate as buffer. None of the mutants showed any significant spectral changes in the pH range tested, at pH values lower than 5 or higher than 9 the wild-type enzyme begins to precipitate and it is impossible to extrapolate accurately any spectral changes once a certain amount of protein aggregation occurs, due to turbidity in the medium. For this reason, the pH range 5 to 9 was used. It is possible that the pKₐ of the K264 and H264 residues within the polypeptide has remained close to its pKₐ in free solution and the pH range tested (and tolerated by the protein) has not been broad enough to protonate/deprotonate the relevant side chains and weaken and/or break the K264 or H264 ligation to the distal position of the heme iron in a significant proportion of the molecules. The lack of any significant spectral changes in the A264Q mutant over the pH 5-9 range may also suggest that lack of any ionization of
Chapter 4 Characterisation of A264K/H/Q P450 BM3 mutants

the Q264 side chain or of neighbouring residues occurs in this pH range and that gross changes in coordination state do not occur.

4.2.8 Redox Potentiometry

The reduction of the wild-type BM3 heme domain in the substrate-free and substrate-bound forms and the corresponding redox potentials are shown in Figure 3.19 and Figure 3.21 of Chapter 3 and discussed therein. The heme reduction potential for each of the mutants was calculated in the same manner as the wild-type enzyme, and data are shown below.

![Reduction of A264K heme domain](image)

**Figure 4.17 Reduction of A264K heme domain**

Main figure: The red spectrum is the starting spectrum, containing ~8 μM A264K heme domain and has a Soret maximum at 424 nm. The green spectrum was the final reduction spectrum collected, it has a split Soret maximum, with species at 424 nm and 445 nm. The spectra shown in black were collected at sequential stages of the reduction. Shown inset is the absorbance change at 445 nm, plotted against the corresponding potential, and fitted to a one electron Nernst equation, giving a reduction potential of -420 mV ± 8 mV.
The spectra of the A264K mutant are not like those of the wild-type enzyme in either the oxidised or reduced form. As was discussed above, the oxidised A264K spectrum suggests the presence of a lysine ligation to the distal position of the heme iron in addition to the remaining proximal cysteine. On reduction, the Soret maximum shifts towards 445 nm, rather than to 410 nm as the wild-type enzyme does. In addition, a distinctive shoulder appears at around 400 nm as reduction progresses and the position of the original Soret band blue shifts to ~ 420 nm. On reduction there also appears to be some heterogeneity in the sample with the α and β-bands splitting into 3 distinct bands with peaks at 529 nm, 558 nm and 568 nm. The spectral changes suggest that the enzyme converts from a predominant single species in the oxidised form to multiple species in the reduced form. This will be discussed further in relation to the A264H reduction potential shown below in Figure 4.18. The redox potential for A264K has been calculated at -420 ± 8 mV, while the substrate-free wild-type enzyme has a redox potential of -392 ± 5 mV, significantly more positive than the potential of the A264K mutant. The negative shift in redox potential can be attributed to the strong ligation between the K264 side chain and the heme iron, forcing the heme iron further towards low-spin and possibly increasing electron density on the heme iron. It should be emphasised also here that the spectral data presented in Figure 4.16 above suggest that there is heterogeneity of species in the reduced form of the enzyme, and that the reduction process has not progressed to completion within the accessible range using dithionite as the reductant. Thus, the predicted heme iron reduction potential in the A264K mutant should be treated with a degree of caution and viewed as an upper limit, i.e. the “real” potential may be significantly more negative.
Chapter 4 Characterisation of A264K/H/Q P450 BM3 mutants

Figure 4.18 Reduction of A264H heme domain

Main figure: The green spectrum shows the fully oxidised form of the enzyme (~9 μM), with a Soret maximum at 426 nm at a potential of -28 mV. The red spectrum shows the final reduction spectrum collected and corresponds to a potential of -462 mV, the Soret maximum is at 428 nm with α and β-bands at 558 nm and 530 nm respectively. Shown in inset is the plot of absorption change at 426 nm versus applied potential. Data were fitted to the one electron Nernst equation, giving a potential of -412 ± 8 mV.

Comparisons between the reduction of wild-type BM3 heme domain and A264H heme domain show marked differences. The A264H mutant has a different reduced spectrum to the wild-type enzyme, with its Soret maximum moving to 428 nm and the α and β-bands shifting to a slightly longer wavelength and increasing in intensity, rather than merging as they do in the wild-type enzyme. These spectral differences suggest that the enzyme is undergoing reduction from ferric to ferrous heme iron, that (unlike with the A264K mutant) a single reduced form may be present, and that the histidine probably remains coordinated to the heme iron in the reduced form. Work carried out by Perera et al. involved analysis of neutral thiol heme iron ligands (164). They created mutants of myoglobin with no 5th ligand and bound tetrahydrothiophene (THT) or cyclopentanethiol (CSPH) to the heme in the proximal position to mimic cysteine thiol ligation. Studying these novel adducts gave a shift in Soret maximum to 428 nm, which they attributed to ferrous 5-coordinate heme with a neutral proton-
bearing thiol as proximal ligand, the spectra also showed $\alpha$ and $\beta$-bands at $\sim 530$ nm and $559$ nm (164). Comparison between these data and the reduction of the A264H BM3 mutant suggests that is the A264H mutant may also undergo a transition from thiolate to thiol proximal ligation in the ferrous form. The thiolate ligation usually seen in P450 is essential for forcing electron density towards the heme iron and enabling oxygen scission and the productive P450 catalytic cycle (11). The identical shifts in Soret maximum seen in the ferrous thiol ligated myoglobin with no 6th ligand and ferrous A264H suggest that the H264 residue is no longer ligating to the heme iron but it follows that loss of the H264 ligation would leave a water-ligated P450 BM3 like the wild-type enzyme, which shows normal thiolate ligation in the ferrous form. If the A264K reduction spectra shown in Figure 4.17 are also compared, it can be seen that a proportion of the most reduced spectrum appears to be in a similar spectral form as the reduced A264H mutant. This may imply that neutral thiol ligation to the heme iron occurs in a proportion of the reduced species of A264K enzyme. The heterogeneity is potentially due to mixtures of reduced species in which the lysine is on or off, and in which the thiolate is deprotonated or protonated. The protonation of the thiolate proximal ligand to form a neutral thiol ligand may be possible due to alterations in the heme electronic organisation in the H264- and K264-ligated states may affect the thiolate such that protonation becomes favourable.

As the A264K and A264H mutants may form a neutral thiol ligand in the ferrous form, the effects of pH on the reduced spectra of the mutants were investigated to see if the proportions of the heterogeneous ferrous adducts could be altered, particularly in the case of the A264K mutant. Although no significant changes were seen by varying the pH of the mutant enzymes in the ferric, substrate-free form, it was considered that the change from ferric to ferrous heme might have more significant effects on the heme system and its ligands. Spectra were collected for the A264K and A264H heme domain in the presence of excess sodium dithionite in an anaerobic environment between pH 5 and pH 9, using 100 mM KPi with 10 % glycerol (v/v) as buffer.
Chapter 4 Characterisation of A264K/H/Q P450 BM3 mutants

Figure 4.19  **Effect of pH on reduced A264K heme domain**
Each spectrum shows ~ 2.5 μM A264K heme domain following incubation with excess sodium dithionite. The red spectrum collected at pH 7 shows mixed species with its Soret maximum at 422 nm and 447 nm. The green spectrum collected at pH 6 has its Soret maximum at 422 nm with α and β-bands at 558 nm and 527 nm. The blue spectrum was collected at pH 9 and has its Soret maximum at 447 nm and α and β-bands at 568 nm and 537 nm.

As can be seen above in Figure 4.19, pH has had an effect on the reduced spectrum of the A264K mutant. The spectrum collected at pH 7 shows mixed species which, as discussed above, may be attributed (at least in part) to ferrous thiol/lysine ligated heme iron and ferrous thiolate/lysine ligated heme iron. Spectra collected at basic pH values showed a shift in the equilibrium with its Soret maximum at 447 nm, suggesting a larger proportion of ferrous thiolate/lysine ligated heme iron. Shifting to more acidic pH values resulted in a shift in the equilibrium to a spectrum with maxima at values identical to those observed in the reduction of A264H heme domain as shown in Figure 4.18, which may possibly be attributed to a thiol-ligated heme iron with lysine as distal ligand. These findings will be discussed further in relation to MCD data obtained in section 4.2.10.
Figure 4.20  **A264Q heme domain reduction**

The red spectrum shown is that of the fully oxidised enzyme (~7 μM) with a Soret maximum at 418 nm and corresponding to a potential of 54 mV. The spectrum shown in green was the final one collected. It has a Soret maximum at 408 nm and corresponds to a potential of -446 mV. Shown in inset is the absorbance at 417 nm plotted against the corresponding potential. The data were fitted to a one electron Nernst equation, giving a redox potential of -396 ± 5 mV.

The A264Q heme domain reduced spectrum suggests that the mutation is not having the same effects on the ligation of the heme iron as the A264K and A264H mutants, instead displaying spectral changes upon reduction similar to those of the wild-type enzyme, with the Soret maximum shifting towards 410 nm on reduction and fusion of the α and β-bands. The calculated redox potential is also similar to the -392 ± 5 mV for the substrate-free wild-type enzyme, suggesting that the A264Q mutant has not had significant effects on the reduction of the heme iron and that the P450 is likely to be predominantly in the water-ligated form in the oxidised state.
Chapter 4 Characterisation of A264K/H/Q P450 BM3 mutants

4.2.9 EPR

EPR spectra were collected for each of the mutants, as they provide a sensitive method for the analysis of ligation and oxidation states of heme proteins, as was discussed in 3.2.8 Chapter 3. EPR spectra were collected by Dr Harriet Seward (University of Leicester) at the Department of Chemistry, University of East Anglia.

![EPR spectra of A264K, A264H and A264Q heme domain mutants](image)

**Figure 4.21** EPR spectra of A264K, A264H and A264Q heme domain mutants

All spectra were collected at 9.67 GHz, 2 mW and 10.8 K, with a protein concentration ~ 500 µM. Shown in red is the A264K mutant, in green the A264H mutant and in blue the A264Q mutant. Relevant g-values are assigned in each spectrum.

<table>
<thead>
<tr>
<th></th>
<th>$g_x$</th>
<th>$g_y$</th>
<th>$g_z$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>2.42</td>
<td>2.26</td>
<td>1.92</td>
</tr>
<tr>
<td>Wild-type + substrate</td>
<td>8.18</td>
<td>2.26/3.44</td>
<td>1.66</td>
</tr>
<tr>
<td>Wild-type + 4PheIm</td>
<td>2.57</td>
<td>2.26</td>
<td>1.86/1.85</td>
</tr>
<tr>
<td>A264K</td>
<td>2.475</td>
<td>2.25</td>
<td>1.915</td>
</tr>
<tr>
<td>A264H</td>
<td>2.498</td>
<td>2.25</td>
<td>1.887</td>
</tr>
<tr>
<td>A264Q</td>
<td>2.475</td>
<td>2.25</td>
<td>1.903</td>
</tr>
</tbody>
</table>
Comparison of the EPR spectra shown in Figure 4.21 (and summarised in Table 4.2) with data obtained for the wild-type enzyme shows marked differences in each of the mutants. As was shown in Figure 3.23 of Chapter 3 the EPR spectrum of wild-type BM3 heme domain has g-values of 2.42 (g\textsubscript{z}), 2.26 (g\textsubscript{y}) and 1.92 (g\textsubscript{x}) in the oxidised low spin form, shifting to 8.18 (g\textsubscript{z}), 3.44 (g\textsubscript{y}), 2.26 (g\textsubscript{y}) and 1.66 (g\textsubscript{x}) in the substrate bound form and having major bands at 2.57(g\textsubscript{z}), 2.26 (g\textsubscript{y}) and 1.86/1.85 (g\textsubscript{x}) in the 4-phenylimidazole bound form. Comparisons between the cysteine/histidine or cysteine/imidazole-ligated heme proteins heme 2 of SoxA (233) and imidazole-ligated P450 cam (150) and P450 BM3 show clearly that the g-values obtained for the A264K and A264H mutants are shifted compared to the wild-type enzyme because of the presence of nitrogenous ligands to the heme and with cysteine proximal ligation maintained, confirming data shown earlier which suggested strong nitrogen ligation. The A264Q mutant g-values are also shifted with respect to the wild-type enzyme, suggesting that the glutamine residue is interacting with the heme at the low temperatures (10 K) at which the EPR measurements were carried out. However, at this stage no firm conclusions can be drawn as to whether the nitrogen or oxygen atoms from the glutamine might be coordinating the heme iron, or whether another interaction (perhaps via the water ligand) occurs.

4.2.10 MCD

MCD spectra were collected for each of the mutants in both the near infrared region and in the UV-visible region by Dr Harriet Seward (University of Leicester) at the Department of Chemistry, University of East Anglia.
Chapter 4 Characterisation of A264K/H/Q P450 BM3 mutants

Figure 4.22 Near-UV-visible region MCD of A264K, A264H, A264Q mutants and wild-type BM3 heme domains

Shown in black is the wild-type heme domain, in red A264K, green A264Q and blue A264H. All spectra were collected of protein at a concentration of 100 μM.

The A264K, A264H and A264Q mutants all show clear shifts in near-UV visible MCD band position and relative intensity with respect to the wild-type enzyme. The general spectral pattern obtained for each of the mutants is indicative of low-spin, ferric heme. In the A264K and A264H mutants the CT$_2$ band has shifted to 583 nm and 587 nm respectively and increased significantly in intensity. Although this is normally indicative of high-spin heme iron, in this case it is known from previous data (and from data shown below) that there is no high-spin content in the A264K or A264H samples. This signal is therefore due to the presence of the K264 or H264 ligations to the heme iron. The general spectral patterns in the A264K and A264H mutants show the Soret derivative cross over at 425 nm, which is again indicative of nitrogen ligation to the heme iron (233). The A264Q mutant spectrum is slightly shifted compared to the wild-type enzyme, but it is unclear whether the shifts are due to novel ligation through either the amide nitrogen or oxygen, or whether there is no physical
coordination but the glutamine residue otherwise affects the heme whilst the distal water molecule is retained.

![Near infrared MCD spectra of wild-type, A264K, A264H and A264Q BM3 heme domains](image)

All spectra were collected in a 1 mm pathlength cuvette. The spectrum shown in black is that of 300 μM wild-type enzyme with its maximum at 1078 nm. Shown in cyan is 500 μM wild-type following the addition of saturating amounts of arachidonate, with its maximum shifted to 834 nm. Shown in red is A264K at a concentration of ~90 μM with its maximum at 1104 nm. The A264H mutant is shown in blue at a concentration of ~120 μM with its maximum at 1160 nm, and in green is the A264Q mutant at ~80 μM with its maximum at 1084 nm.

As with UV-visible MCD spectra, there are significant differences between the spectra of the wild-type enzyme and the A264K, A264H and A264Q mutants in the near-infrared region. The maximum seen at 1078 nm in the wild-type enzyme is indicative of low-spin ferric heme, shifting to 834 nm in the high-spin, fatty acid-bound form. Comparison between the A264H MCD spectrum and MCD spectra of the wild-type enzyme with 4-phenylimidazole bound (140) and heme 2 of SoxA (233) strongly suggests that the H264 residue ligates to the distal position of the heme iron, with thiolate ligation at the proximal position. In its native state, CooA has cysteine and proline ligation, with the proline residue being the terminal residue in the polypeptide.
Chapter 4 Characterisation of A264K/H/Q P450 BM3 mutants

chain and with ligation through its nitrogen. Comparison between MCD data obtained for CooA, which showed a spectral maximum at 1120 nm in the near-infrared region (243) and the data shown in Figure 4.23 for the A264K mutant (which has its spectral maximum at 1104 nm) indicates clearly that the K264 residue ligates to the heme iron. The spectrum obtained for the A264Q mutant is more similar to the wild-type enzyme than to either the A264K or A264H spectra. Data obtained for the A264E mutant, shown in 3.2.9 Chapter 3 (225), show that in the near-infrared region the mutant has its spectral maximum at 1084 nm both in the presence and absence of substrate. As was discussed in Chapter 3, a proportion of the enzyme has E264 ligation to the distal position of the heme iron, and following addition of substrate the proportion of E264 ligation increases. The spectral maximum is also found at 1084 nm in the A264Q near-infrared MCD spectrum. This suggests that at least a proportion of the enzyme may have Q264 ligation to the heme iron at the distal position through the glutamine oxygen, rather than through the nitrogen.

As was shown in section 4.2.8, reduced spectra collected for the A264K and A264H mutants suggest that the enzyme is no longer entirely thiolate-ligated and that there may be a proportion of thiol ligation, at least in the reduced forms. To further assess this, MCD spectra of the A264K and A264H mutants were collected in partially reduced forms. Excess sodium dithionite was added to 500 µM A264K or A264H heme domain, and incubation was carried in an anaerobic environment for 1 hour prior to spectra collection in order to maximise the proportion of the enzymes reduced to their ferrous forms.
Chapter 4 Characterisation of A264K/H/Q P450 BM3 mutants

Figure 4.24 Near-UV-visible region MCD of oxidised and reduced A264K heme domain

Shown in red is the oxidised spectrum of A264K heme domain and shown in blue is the spectrum of reduced A264K heme domain. The reduced spectrum was generated by subtracting the oxidised spectrum from the partially reduced spectrum, as full reduction could not be achieved using sodium dithionite.
Chapter 4 Characterisation of A264K/H/Q P450 BM3 mutants

Figure 4.25 Near-UV-visible MCD spectra of oxidised and reduced A264H heme domain
Shown in blue is the spectrum of reduced A264H heme domain, generated by subtracting the oxidised contributions from the spectrum collected of maximally reduced A264H heme domain. The spectrum has its Soret maximum at 426 nm and a derivative species at 557 nm. The spectrum shown in red is of oxidised A264H with its Soret maximum at 414 nm. The region between 450 nm and 850 nm has been multiplied by 5 for clarity.

The Soret maximum seen at 415 nm in the oxidised near-UV-visible MCD spectra of both the A264K and A264H mutants shifts to 426 nm upon reduction. Comparison of these data and data previously obtained for P450 cam(35) and the H93G mutant of myoglobin with thiol and thiolate axial ligations (164) suggest that the shift in Soret maximum to 426 nm is indicative of thiol ligation. Spectra were also generated by Perera et al. (164) of the inactive ferrous P420 form of P450 2B4, and this also shows thiol ligation in its near-UV-visible MCD spectrum with spectral maxima in the same positions as those seen for the other examples. Ferrous thiolate-ligated P450 LM2 near-UV-visible MCD spectra have been collected (164). These have their Soret maximum at shorter wavelength and do not show the presence of any derivative form at 557 nm. It can therefore be concluded that the A264H and A264K mutants become...
(at least partially) protonated when their heme iron is reduced from ferric to ferrous, resulting in thiol, rather than thiolate, ligation. The P420 form of the cytochromes P450 are inactive, and the inactivity is thought to be due to the inability of the thiol-ligated P420 form to catalyse reductive scission of bound molecular oxygen, with alteration to thiol ligation leading to the lack of a strong electron “pushing” effect obtained in the thiolate-ligated form(11).

4.2.11 Resonance Raman

Resonance Raman spectra were obtained for wild-type heme domain and the A264K/H/Q mutants in collaboration with Dr Rachael Littleford (Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow) using a 406 nm laser source in the low, mid and high frequency regions. All spectra were collected using 50 μM BM3 as described in the Methods. All assignments were made on the basis of work published by Smith et. al. (218).
Chapter 4 Characterisation of A264K/H/Q P450 BM3 mutants

Figure 4.26  Wild-type and A264K, Q and H mutant BM3 heme domain resonance Raman spectra
All spectra were collected with ~50 μM BM3 heme domain. Wild-type BM3 is shown in black, and following the addition of 200 μM arachidonic acid in red. Spectra collected for the A264K, Q and H mutants are shown in green, blue and cyan respectively. The Y-axis of each spectrum has been offset for clarity, and the v4 band is indicated.

Resonance Raman provides a sensitive method for detecting changes in the oxidation state and spin state of the cytochromes P450 (219). Addition of the substrate arachidonate to the A264K and A264H mutants did not affect resonance Raman spectra, confirming that the A264K and H mutants are not capable of the low- to high-spin transition seen on binding of substrate to the wild-type enzyme. Minor changes were seen with the A264Q mutant, these are shown below in Figure 4.28. The v4 band
Chapter 4 Characterisation of A264K/H/Q P450 BM3 mutants

indicated on the resonance Raman spectra shown in Figure 4.26 is an oxidation state marker, found at 1372 cm\(^{-1}\) in wild-type ferric P450 BM3. Each of the A264K/Q/H mutants also have their \(v_4\) band at 1372 cm\(^{-1}\) indicating that their heme iron is in the ferric form, as was expected from UV-visible spectroscopy, EPR and MCD spectra. A number of significant changes are seen between the wild-type and mutant spectra in the high-frequency region; this is shown in more detail in Figure 4.27.

Figure 4.27 High frequency resonance Raman spectra of A264K and wild-type BM3
Shown in green is substrate-free wild-type P450 BM3, and in cyan following the addition of arachidonate. The A264K mutant is shown in red. All proteins were at a concentration of \(~ 50\ \mu\text{M}\). Curve fitting was carried out on the A264K mutant with the curves shown as black lines and the residual spectrum from subtraction of the curve fit from the original spectrum shown in blue. Assignments, made on the basis of curve fit data, are indicated (218).
Chapter 4 Characterisation of A264K/H/Q P450 BM3 mutants

Figure 4.28 High-frequency resonance Raman spectra of A264Q and wild-type P450 BM3

Shown in green is the spectrum for wild-type P450 BM3, in red that for the A264Q mutant, and in magenta the A264Q mutant spectrum following the addition of substrate at a concentration of ~ 50 μM. Curve-fitting is shown for the substrate-free A264Q mutant in black and residual spectrum shown in blue calculated by subtraction of the peak fit from the A264Q spectrum. Assignments, based on the curve fitting and on work by Smith et al. (218), are indicated.
Chapter 4 Characterisation of A264K/H/Q P450 BM3 mutants

Figure 4.29 High-frequency resonance Raman spectra of A264H and wild-type P450 BM3
Shown in green is the spectrum for wild-type P450 BM3, in red the A264H mutant spectrum, and in magenta the wild-type spectrum following the addition of substrate at a concentration of ~200 μM. Curve-fitting is shown for the substrate-free A264H mutant in black, and the residual spectrum shown in blue is calculated by subtraction of the peak fit from the A264H spectrum. Assignments, based on the curve fitting and work by Smith et al. (218), are indicated.

Table 4.3 Wild-type, A264K/H/Q principle resonance Raman spectral features

<table>
<thead>
<tr>
<th></th>
<th>(v_4) (cm(^{-1}))</th>
<th>(v_{11}) (cm(^{-1}))</th>
<th>(v_3) (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>1372</td>
<td>1565</td>
<td>1495</td>
</tr>
<tr>
<td>A264K</td>
<td>1371</td>
<td>not present</td>
<td>1495</td>
</tr>
<tr>
<td>A264H</td>
<td>1377</td>
<td>not present</td>
<td>1495</td>
</tr>
<tr>
<td>A264Q</td>
<td>1371</td>
<td>1567</td>
<td>1495/1485</td>
</tr>
</tbody>
</table>

The high frequency region of resonance Raman spectrum is particularly useful for gaining information on the spin-state of the enzyme (244). In particular the \(v_3\) band is diagnostic of spin-state changes. In the high-spin form the wild-type enzyme splitting
of the $v_3$ band can be seen, with peaks at both 1495 cm$^{-1}$ and 1485 cm$^{-1}$. With the exception of substrate-bound A264Q, each of the mutants has their $v_3$ band at 1495 cm$^{-1}$, indicating that they are almost entirely low-spin. Substrate-bound A264Q shows a small shoulder at 1485 cm$^{-1}$, confirming that a small proportion of the enzyme is in the high-spin form. The change in intensity of the $v_3$ band is indicative of nitrogen ligation to the heme iron (218) and confirms that the A264K and A264H mutants both have lysine and histidine axial ligation in addition to the cysteinate P450 ligation. The wild-type enzyme, in both the substrate-free and substrate-bound forms, and the A264Q enzyme contain a $v_{11}$ band at 1565 cm$^{-1}$, which has decreases in intensity in the A264H and A264K mutants. The $v_{11}$ band reports on the in-plane asymmetry of the heme ring (218). The doming of the heme ring in the presence of a distal water ligation promotes this band. The presence of lysine or histidine ligation has therefore reduced the asymmetry of the heme ring, making it more planar. Thus, the absence of the $v_{11}$ band again confirms the presence of lysine and histidine as distal ligands in the A264K and A264H mutants, respectively. Interestingly, the A264Q mutant shows a slight change in intensity of the $v_{11}$ band, suggesting that there is either in an equilibrium where a proportion of the enzyme contains nitrogen ligation to the heme iron from the glutamine, or that the close proximity of the glutamine affects the heme plane but does not displace the distal water. Alternatively, the oxygen group of the Q264 residue may interact with the heme iron, but does not form a strong enough ligation to change the planarity of the heme ring as significantly as with the K264 or H264 ligations.

Resonance Raman is a particularly useful technique for the analysis of the heme plane, but does not provide as much information on the axial ligations to the heme iron, and therefore differences between the lysine and histidine ligations cannot be clearly discerned with resonance Raman spectroscopy.

### 4.2.12 Steady-state kinetic analysis

Although no spin-shifts were detected on substrate-binding to the A264K and A264H mutants, and only very limited optical changes observed on substrate addition to the
A264Q mutant, steady-state kinetic measurements were made to assess the rate of substrate-dependent NADPH oxidation by wild-type and mutant flavocytochromes P450 BM3, using the substrates arachidonate and laurate. In Chapter 3 it was shown that the A264E mutant retained significant levels of fatty acid hydroxylase activity, despite the fact that substrate binding induced coordination of the heme iron by the E264 side chain. Thus, studies of the A264E mutant indicated that the E264 ligand to the iron could be effectively displaced during turnover. It was of interest to establish whether any similar behaviour was observed with these other mutants – particularly in the cases of A264K/H where there appeared to be near-complete distal heme iron coordination even in the absence of substrate.

A degree of substrate-dependent NADPH oxidation was seen with each of the mutants, but at very low rates compared to those seen with the wild-type enzyme, the rates and $K_M$ calculated are shown in Table 4.4, and laurate-dependent NADPH oxidation by the mutants shown below in Figure 4.30, Figure 4.31 and Figure 4.32.

**Figure 4.30** Lauric acid-dependent NADPH oxidation by the A264K mutant of flavocytochrome P450 BM3

The rates of NADPH oxidation were averaged from three assays at each lauric acid concentration. The resultant data describe a linear dependence of rate on lipid concentration, rather than the hyperbolic dependence seen in the wild-type enzyme. Data have been fitted to a straight line yielding a rate of $3.13 \pm 1.64 \text{ min}^{-1} \mu \text{M}^{-1}$ laurate.
Chapter 4 Characterisation of A264K/H/Q P450 BM3 mutants

Figure 4.31 Lauric acid-dependent NADPH oxidation by the A264H mutant of flavocytochrome P450 BM3

The rates of substrate dependent NADPH oxidation were averaged from 3 assays at each substrate concentration. The data display a linear dependence of NADPH oxidation rate on lipid concentration, rather than a hyperbolic dependence. Data were fitted to a straight line yielding a rate of $0.25 \pm 0.07 \text{ min}^{-1} \mu\text{M}^{-1} \text{laurate}$.

Figure 4.32 Lauric acid-dependent NADPH oxidation by the A264Q mutant of flavocytochrome P450 BM3

The rates of substrate dependent NADPH oxidation were averaged from 3 assays at each substrate concentration. The data display linear dependence of NADPH oxidation rate on lipid concentration rather than hyperbolic dependence. Data were fitted to a straight line yielding a rate of $1.92 \pm 0.17 \text{ min}^{-1} \mu\text{M}^{-1} \text{laurate}$.
Chapter 4 Characterisation of A264K/H/Q P450 BM3 mutants

Table 4.4 Maximal rates of substrate-dependent NADPH oxidation and corresponding $K_M$ values for wild-type and A264K/H/Q flavocytochrome P450 BM3 mutants

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Wild-type $k_{cat}$(min$^{-1}$)</th>
<th>A264K $k_{cat}$</th>
<th>A264H $k_{cat}$</th>
<th>A264Q $k_{cat}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonate</td>
<td>16400 ± 185</td>
<td>754 ± 14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{cat}$ (min$^{-1}$)</td>
<td>1.86 ± 0.46</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_M$ (μM)</td>
<td>5.1 ± 0.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{cat}/K_M$(min$^{-1}$μM$^{-1}$)</td>
<td>3215</td>
<td>3.13 ± 1.64*</td>
<td>0.89 ± 0.21*</td>
<td>400</td>
</tr>
<tr>
<td>Laurate</td>
<td>2770 ± 120</td>
<td>87.4 ± 8.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{cat}$ (min$^{-1}$)</td>
<td>120</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_M$ (μM)</td>
<td>31.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{cat}/K_M$(μM$^{-1}$min$^{-1}$)</td>
<td>31.7</td>
<td>0.36 ± 0.02*</td>
<td>0.25 ± 0.07*</td>
<td>1.92 ± 0.17*</td>
</tr>
</tbody>
</table>

* the values indicated are second order rate constants obtained from the gradient of the line of best fit through the rate versus fatty acid concentration data (in units of μM$^{-1}$min$^{-1}$).

As can be seen in Figure 4.30, Figure 4.31, Figure 4.32 and Table 4.4 the A264H/K mutants showed essentially linear dependence of NADPH rate on fatty acid concentration (with both arachidonate and laurate as substrates), rather than the hyperbolic dependence seen for wild-type P450 BM3. The second order rate constants cited in these cases come from the gradient of the line of best fit through the rate versus substrate concentration data. The linear dependence must reflect the inability of the substrates to saturate the enzymes within the accessible concentration range. This, in turn, may reflect the low proportion of enzyme active sites in which the substrate can bind in a productive orientation (i.e. in which there is not steric obstruction of the heme pocket or direct coordination of the iron by the amino acid side chain). The substrate concentration range used is limited by the substrate solubility, with laurate being soluble to ~950 μM in assay buffer and arachidonate to ~330 μM (with 10 μl of a 33 mM stock solution in the solvent ethanol added to give a final assay volume of 1000 μl at the highest concentration used). However, it is clear that despite the reasons underlying the low fatty-acid dependent NADPH oxidase activity of the A264K/H, their capacity to oxygenate fatty acids must be dramatically lowered with respect to the wild-type enzyme, and also with respect to the A264E mutant. However, it is plausible that the small amount of fatty acid-dependent NADPH oxidation observed could relate
to true oxygenation of fatty acids (this is tested below), as seen for the A264E variant. Although fatty acid binding to A264H/K does not induce increased high-spin content (indeed there are negligible spectral changes observed), fatty acid binding to A264E only served to increase low-spin heme iron content of the heme. However, a further consideration with the A264K/H mutants is (from potentiometric studies) their apparent tendency to form P420 (i.e. protonation of thiolate proximal ligand to thiol) on reduction. This may further compromise their catalytic capacity as fatty acid hydroxylases.

In the A264Q mutant differences are seen in turnover with arachidonic acid and lauric acid. In the case of the former, a clear hyperbolic dependence of NADPH oxidation rate on fatty acid concentration is observed (albeit with $k_{cat}$ substantially lower than wild-type), suggesting high affinity for the fatty acid. However, a linear dependence is seen with lauric acid, with substantially lower turnover rates. It would appear that there are marked differences in A264Q's affinity for these two fatty acids with respect to wild-type (similar or tighter with arachidonate, weaker with lauric acid – a trend which was also seen in substrate binding as shown in section 4.2.5, but that the A264Q variant (unlike the A264K/H enzymes) retains a considerable amount of catalytic activity – at least with arachidonic acid as substrate.

### 4.2.13 Product analysis

Steady-state kinetic analysis showed that each of the mutants had some degree of substrate-dependent NADPH oxidation. It was therefore of interest to assess whether product was being formed, or if turnover was futile, leading to hydrogen peroxide and/or superoxide formation instead. Lauric acid was incubated with wild-type and mutant flavocytochromes P450 BM3 and NADPH for at least 14 hours and the fatty acids and any products extracted as described in the *Methods* section. Mass spectra were then collected for the extracted fatty acids. Positive ionisation mass spectra of isolates following lauric acid incubation with A264K and A264Q mutants are shown in Figure 4.33 and Figure 4.34. The substrate lauric acid has a mass of 199 amu, monohydroxylated product therefore has a mass of 215 amu.
Chapter 4 Characterisation of A264K/H/Q P450 BM3 mutants

Figure 4.33  Mass spectrum of organic phase isolates following incubation of lauric acid with A264K flavocytochrome P450 BM3 and NADPH.
The species at 199 amu corresponds to lauric acid, but other species do not correspond to hydroxylated lauric acid, and instead are contaminants arising from the extraction process which are seen in control samples.

Mass spectra of the isolated fatty acids using the A264H mutant gave the same results as those seen with the A264K mutant, i.e. there was no formation of any mono-hydroxylated product.
Figure 4.34 Mass spectrum of organic phase isolates following incubation of lauric acid with A264Q flavocytochrome P450 BM3
A small amount of product is seen in this spectrum at 215 amu, with a large amount of substrate still remaining (199 amu), and small amounts of other non-specific material seen (as in Figure 4.33).

Wild-type turnover studies with lauric acid are shown in Chapter 3. In these experiments all substrate was converted to product following incubation with NADPH and enzyme for an extended period. Lauric acid is the weakest binding of all the fatty acids tested and has the lowest rates of substrate-dependent NADPH oxidation with the wild-type enzyme. However, the vastly increased solubility of laurate when compared to the other fatty acids makes it a superior substrate to use in these turnover assays, particularly as the addition of solvents (required for solubilisation of longer chain fatty acids) adversely affected assays and since precipitation of the rather insoluble long chain fatty acid substrates leads to false negative results. In addition, laurate is generally converted only to mono-hydroxylated product, simplifying the analysis required. Each assay was allowed to incubate for at least 14 hours, and UV-visible spectra showed that no NADPH remained.

Both the A264K and A264H mutants showed no product formation, this data concurs with data shown previously. The mutant enzymes do not shift from low- to high-spin on addition of substrate and they have partial inactive thiol ligation following reduction. It was therefore expected that the enzymes might be inactive and produce no product, and thus the results above suggest that the slow substrate-dependent
NADPH oxidation observed reflects only non-specific reduction of molecular oxygen. The A264Q mutant has shown shifts to the high-spin state on addition of substrate and substrate-dependent NADPH oxidation, and is capable of forming a small proportion of product following incubation with substrate for over 14 hours.

### 4.2.14 Measurement of uncoupled enzyme turnover

No product formation was seen with the A264K and A264H mutants and very little product was seen with the A264Q mutant, yet steady-state assays showed that the mutant enzymes catalyse the substrate-dependent oxidation of NADPH (albeit at very low rates in the case of the A264K/H mutants). As was discussed in Chapter 1, it is possible for the enzyme to turn over in a futile manner, generating no oxygenated fatty acid product, but still oxidising NADPH and producing hydrogen peroxide, superoxide or water through reduction of molecular oxygen (157,170). The oxidation of NADPH without formation of product suggests that the mutant enzymes may be producing larger amounts of hydrogen peroxide, superoxide or water in uncoupled turnover pathways. Thus, the formation of hydrogen peroxide and superoxide during fatty acid-dependent NADPH oxidation was measured.

In order to measure the levels of hydrogen peroxide and superoxide, a coupled assay was used with the enzymes horseradish peroxidase (HRP), superoxide dismutase (SOD) and the substrate o-dianisidine as described in the Methods section and in section 3.2.13 of Chapter 3.
Table 4.5  Percentage NADPH converted to hydrogen peroxide or superoxide in wild-type and A264K/Q/H mutant flavocytochromes P450 BM3

200 μM NADPH was incubated with ~ 0.2 μM enzyme in the presence and absence of 950 μM laurate. Assays with and without laurate were begun simultaneously and measurements made once all NADPH was oxidised in assays containing substrate.

<table>
<thead>
<tr>
<th></th>
<th>% NADPH converted to H₂O₂</th>
<th>% NADPH converted to superoxide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>substrate-free</td>
<td>+ laurate</td>
</tr>
<tr>
<td>Wild-type</td>
<td>3.3</td>
<td>1.7</td>
</tr>
<tr>
<td>A264K</td>
<td>1.0</td>
<td>1.6</td>
</tr>
<tr>
<td>A264H</td>
<td>1.0</td>
<td>4.3</td>
</tr>
<tr>
<td>A264Q</td>
<td>1.2</td>
<td>2.9</td>
</tr>
</tbody>
</table>

As can be seen there appears to be very little uncoupling in either the wild-type enzyme or any of the mutants. As was discussed in Chapter 3 and shown in work by Munro *et al.* (220), the wild-type enzyme produces very little hydrogen peroxide or superoxide, with most reducing equivalents being used for product formation. The A264K/H/Q mutants also appear to produce very little hydrogen peroxide or superoxide. Assays carried out with the A264K and A264H mutants took significantly longer to reach an end point than the wild-type enzyme, due to the much slower fatty acid-dependent oxidation of NADPH. The apparent low levels of hydrogen peroxide or superoxide formation together with the absence of oxygenated fatty acid product seen in turnover studies in section 4.2.13 is somewhat confusing and apparently contradictory. The fatty acid dependence of NADPH oxidation rate in all cases demonstrates that the process is largely enzyme-catalysed. Thus, it appears likely that radical products (*i.e.* peroxide and superoxide) of A264K/H/Q turnover may be consumed by reaction with the enzyme, preventing their detection by the end point assay. Potentially, some of the NADPH oxidised could also be used to produce water (which would not be detectable by this assay). However, it is clear that since NADPH oxidation is not linked to fatty acid oxygenation in either A264H or A264K enzymes,
the non-specific NADPH-dependent reduction of dioxygen must predominate for these enzymes.

### 4.2.15 X-ray crystallography

X-ray crystallographic studies of the A264K, A264H and A264Q mutants were carried out by Dr Helen Toogood (Department of Biochemistry, University of Leicester). An initial screen based around the conditions used for the generation of the A264E mutant crystals (Chapter 3) was used to find crystallisation conditions for the A264K, A264H and A264Q mutants. Refinement of the screen resulted in the crystal growth conditions detailed in Table 4.6. Also shown in Table 4.6 is the space group which the enzyme occupied in the crystal and the resolution to which the structure was ultimately refined.

<table>
<thead>
<tr>
<th>Crystallisation conditions</th>
<th>Space group</th>
<th>Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>A264K 100 mM cacodylate pH 6.0, 160 mM MgCl₂, 16 % PEG3350</td>
<td>P₂₁2₁2₁</td>
<td>2.4 Å</td>
</tr>
<tr>
<td>A264H 100 mM cacodylate pH 6.0, 130 mM MgCl₂, 18 % PEG3350</td>
<td>P₂₁</td>
<td>1.9 Å</td>
</tr>
<tr>
<td>A264Q 100 mM cacodylate pH 6.0, 160 mM MgCl₂, 16 % PEG3350</td>
<td>P₂₁2₁2₁</td>
<td>2.1 Å</td>
</tr>
</tbody>
</table>

Data collection was carried out at the European Synchrotron Radiation Facility (Grenoble, France) and structures were solved via molecular replacement using the structures of wild-type and mutant P450 BM3 heme domain (PDB codes 1SMI, 1FAG, 1JPZ and 1BU7) as search models.

The A264K mutant was found to contain 2 molecules in the asymmetric unit, both of which are in the same conformation. The K264 residue side chain coordinates to the heme iron in the distal position, with C400 remaining as proximal ligand, as shown below in Figure 4.35.
Chapter 4 Characterisation of A264K/H/Q P450 BM3 mutants

Figure 4.35 A264K mutant heme domain crystal structure
Both images show the K264 residue, heme and the other important residues F87, T260 and C400, with residues labelled in the image on the left. The image on the right also shows the secondary structure in the region. The closest contacts between F87, T260 and K264 are represented as dashed green lines on the image on the left, F87 lies 5.06 Å from T260 and 4.53 Å from K264, T260 and K264 are separated by 5.17 Å. Images generated using Pymol (245).

Although there is no substrate present the overall structural fold of the A264K mutant is the same as the substrate-bound wild-type structure and the A264E structure (as discussed in Chapter 3).

Like the A264K mutant crystals, 2 molecules were found in the asymmetric unit of the A264Q mutant crystals, with both molecules being identical. There was, however, dual occupancy within the region around the Q264 residue, with density showing both Q264 side chain ligation to the heme iron and Q264 also resting against F87 as shown in Figure 4.36 and Figure 4.37.
The active site of the A264Q mutant shows dual occupancy in a number of the I-helix residues, including Q264. The 2 identifiable forms are shown. The Q264 residue side chain can be seen ligating to the heme iron in panel B (via the amide nitrogen) and sitting against F87 in panel A (with oxygen from a water molecule coordinating the heme iron instead). T260 is also shown. In the ligating position there is 4.46 Å between Q264 and F87 and 7.56 Å between Q264 and T260. In the non-ligating position there is 3.4 Å between Q264 and F87 and 5.7 Å between Q264 and T260. Images generated using Pymol (245).
As can be seen in Figure 4.36 the 2 positions occupied by the Q264 residue are very similar to the position occupied by the E264 residue shown in Chapter 3, with the Q264 residue either sitting against F87 or ligating directly to the heme iron. Interestingly it can also be seen that the ligation occurs through the nitrogen group of the glutamine residue, and not the oxygen, as was suggested by MCD. However, since a proportion of the enzyme retains water ligation the solution state MCD signal observed was likely a mixed signal composed of both water and glutamine coordination. The density around the heme and Q264 residue, shown in Figure 4.37, reveals a number of the problems associated with solving the structure of the dual occupancy Q264 mutant, and it is possible that there is a third form of the Q264 enzyme with the Q264 residue occupying a position between the position seen in either of the other states, or even that there is another water molecule in a non-ligating position and resting against Q264. However, the resolution of the structure is not sufficiently high to resolve completely the heterogeneity.
Chapter 4 Characterisation of A264K/H/Q P450 BM3 mutants

Figure 4.38 A264H structure active site
Shown in both images is the A264H mutant of P450 BM3. The heme group can be seen in red with cysteine 400 below ligating to the heme iron above the heme plane. H264 can be seen, also ligating to the heme iron. Also shown are F87 and T260. The closest contacts between F87, T260 and H264 are indicated. H264 is 3.7 Å from F87 and 4.82 Å from T260, while T260 and F87 are 3.41 Å from one another. The right hand panel also shows the secondary structure around the heme group, with T260 and H264 both in the I-helix and F87 in the loop region. Image generated using Pymol (245).

The A264H crystal structure shows that the H264 residue is ligated to the heme iron in all molecules of the asymmetric unit, as was expected from spectroscopic data shown earlier. Substrate-bound wild-type P450 BM3 overlaid with the A264K/H/Q structures is shown in Figure 4.39 and in Figure 4.40, along with substrate-free wild-type P450 BM3 and the A264K/H/Q mutant structure.
Chapter 4 Characterisation of A264K/H/Q P450 BM3 mutants

Figure 4.39 Palmitoleic acid-bound wild-type P450 BM3 structure overlaid with A264K/H/Q mutant structures
Shown in orange is wild-type P450 BM3 with palmitoleic acid bound (135) (PDB 1FAG). Shown in green is the A264Q mutant, in blue the A264H mutant and in cyan the A264K mutant. The structures have been overlaid and (with the exception of the A264H) mutant show the same overall fold. Alignment and image generated using Weblab (246).
Comparison between the backbone of the A264K/Q/H mutants and wild-type P450 BM3 in substrate-free and substrate-bound forms, as shown in Figure 4.39 and Figure 4.40, reveals that the A264K and A264Q mutants are found in the same conformation as substrate-bound wild-type P450 BM3, whereas the A264H mutant bears more similarity to the substrate-free wild-type P450 BM3 structure.
Chapter 4 Characterisation of A264K/H/Q P450 BM3 mutants

4.3 Discussion

The work carried out in this chapter has shown successful generation of the A264K/Q/H mutants of P450 BM3 in both the heme domain and flavocytochrome constructs. Each of the mutants have been extensively spectroscopically and structurally characterised using a variety of techniques. Analysis of the A264E mutant (Chapter 3) showed that the E264 residue ligates to the distal heme iron position to a limited extent in the absence of substrate and then completely in the presence of substrate. It was therefore the aim of this section of work to generate and characterise a number of other mutants at the 264 position, in which replacement residues were those that might form ligations to the heme iron and therefore generate either novel heme iron ligation states or ligation states which are rarely found in nature. Each of the A264K/Q/H mutants showed ligation between the novel 264 residue side chains and the heme iron to varying degrees, and also showed a number of other interesting and novel features.

The H264 and K264 residues formed tight ligations to the heme iron in the distal position. The histidine and lysine heme iron coordination remains largely unaffected by changes in the heme environment; including as a result of variations in pH, temperature and ionic strength. It was not possible to displace the ligations between K/H264 and the heme iron using carbon monoxide (for the ferrous enzymes), nitric oxide or the inhibitors sodium cyanide or 4-phenylimidazole, each of which ligates to the heme iron in the distal position in the wild-type enzyme (albeit with varying affinities). These mutants also display no significant spectral changes on titration with fatty acid substrates, are apparently incapable of undergoing spectral transition indicative of a shift from low- to high-spin heme iron, and are not catalytically active with respect to NADPH-dependent fatty acid oxygenation. Although there is some stimulation of NADPH oxidation by fatty acids in A264K/H enzymes, this is almost certainly a result of non-specific reduction of dioxygen without lipid hydroxylation.

This work has also resulted in a number of other interesting findings. Under reducing conditions the A264H and A264K mutants do not produce the optical changes observed on reduction of the ferric heme iron to the ferrous form in wild-type P450 BM3. Instead, the shifts of the Soret band and the peculiar changes in the visible region observed on reduction of the A264K/H heme iron are suggestive of the
protonation of the proximal thiolate ligand to a thiol on reduction of the heme iron (164).

In the A264H mutant there appears to be no change in the strongly positive histidine ligation on reduction of the heme iron, but the A264K mutant shows some pH dependency in the spectroscopic properties of its reduced species suggesting that there is an equilibrium between thiolate and thiol ligation, and possibly also changes in the charge of the K264 residue. The A264Q mutant did not result in ligation between the Q264 side chain and the heme iron in all circumstances. A limited amount of substrate-induced spin-shift is seen and the mutant is capable of a limited amount of product formation, indicating strongly that native P450 BM3-like Cys-aqua coordination is present in some of the species in solution. However, spectroscopic and crystallographic analysis has shown that the Q264 residue does ligate to the heme iron in a proportion of the enzyme, with the Q264 residue resting against F87 in the other proportion of the enzyme, as was seen with the A264E mutant in Chapter 3. The results have allowed characterisation of a set of enzymes containing novel heme iron ligation states, namely Cys/Glu, Cys/His and Cys/Lys, generating spectroscopic standards which will be of use in the spectroscopic identification and characterisation of novel heme-containing proteins with similar or identical types of heme iron ligation. In particular, the analysis by MCD and EPR techniques help to contribute to the “spectroscopic ruler” by which a library of specific g-values (EPR) and positions of the NIR CT band (MCD) enable the identification of the ligands to heme iron in novel proteins based on pre-existing data set for structurally verified cytochromes.

Analysis of the crystal structures has shown a number of further interesting features. The A264K and A264H mutants have ligation between the H264 and K264 amino acid side chains and the heme iron in the distal position, while the A264Q mutant shows dual occupancy with one form having Q264 side chain heme iron ligation, as was expected from spectroscopic analysis, while the other form is aqua ligated. In common with the A264E structure shown in Chapter 3, the A264K and A264Q mutants adopt the same conformation as wild-type substrate-bound (SB) P450 BM3, although they do not contain fatty acid substrate. The A264H mutant is not in the substrate-bound conformation and is instead found in a similar conformation to the
substrate-free wild-type enzyme (SF). Previously it has been hypothesised that binding of substrate causes displacement of the water molecule distal to the heme iron and that the motion of this water induces a spectral change from the SF form to SB form (147). As was discussed in Chapter 3, the structure of the A264E mutant heme domain was found to be in the SB conformation in the absence of substrate, with the distal water present in molecules for which the E264 side chain was not ligated to the iron. The A264H structure further challenges this theory, suggesting that wild-type P450 BM3 may occupy multiple conformational species in solution. These species likely exist in an equilibrium dominated by the SF form in the absence of substrate and the SB form in the presence of substrate, with multiple other forms possible, including the species apparently stabilised by the A264H mutation.
Chapter 5 Characterisation of A264C/M mutant forms of flavocytochrome P450 BM3 and its heme domain: Novel P450 heme ligation states
5.1 Introduction

In the preceding Chapters 3 and 4, it has been shown that mutagenesis of the A264 residue of P450 BM3 to either amino acids with functional groups containing oxygen (i.e. glutamate) or nitrogen (i.e. histidine, glutamine and lysine) atoms in their side chains can result in ligation of the mutated 264 residue side chain to the distal position of the heme iron, generating the little studied or novel ligation states Cys/Glu, Cys/Lys, Cys/His and Cys/Gln. As yet no cytochromes have been isolated with sulphur ligations to both distal and proximal positions, where cysteine forms one of those ligations. It is therefore of interest to assess whether mutagenesis at position 264 to either methionine or cysteine can lead to a Cys/Cys or Cys/Met ligated iron and what implications these ligation states would have on the spectroscopic features of the heme itself and the catalytic features of the enzyme as a whole.

Although Cys/Cys or Cys/Met heme ligation have not been discovered to date, a large number of cytochromes have been isolated with either cysteine or methionine ligation. Cysteine ligated cytochromes are principally confined to the cytochromes P450 and nitric oxide synthases, which are discussed in general terms in Chapter 7, and SoxAX, CooA and cystathione β-synthase, which have been discussed in the introduction to Chapter 4.

There are a number of examples of cytochromes with methionine ligated heme iron. The largest group are a number of members of the c-type cytochrome family. As was discussed in Chapter 1, the c-type cytochromes contain a heme group ligated via the porphyrin macrocycle to two cysteine residues in the consensus sequence CXXCH, where the histidine forms the proximal heme iron ligand (12). One example of a c-type cytochrome with methionine as an axial ligand is cytochrome c₁, which is part of the mitochondrial cytochrome bc₁ complex. The cytochrome bc₁ complex is a transmembrane oligomeric protein which is complex III of the respiratory chain, coupling the oxidation of ubiquinol to the translocation of protons across the transmembrane space (via the ubiquinol itself – the so-called “Q-cycle”) and the reduction of cytochrome c, which then passes electrons to cytochrome c oxidase (complex IV). The cofactors contained within cytochrome bc₁ are two b-type hemes, one with low potential (b₁) and one with high potential (b₁), a 2Fe2S cluster and a
cytochrome c (c1) (247). The mechanism, known as the Q cycle, begins with the oxidation of ubiquinol and concomitant release of protons to the intermembrane space, with one electron passed to heme bL and the other to the iron sulphur centre. The reduced heme bL then passes the electron to the heme bH centre which in turn passes it to a ubiquinone binding site where ubiquinone is reduced and protons are transported across the outer membrane. The iron sulphur protein reduces cytochrome c1 which is in turn oxidised by cytochrome c in the intermembrane space (248). Cytochrome c1 is a c-type cytochrome with a covalently ligated heme, histidine proximal ligation and methionine distal ligation (249). The importance of the distal ligand methionine has been investigated by Gray et al., who mutated the axial methionine to a leucine. The M183L mutant was created in cytochrome c1 from the photosynthetic bacteria *Rhodobacter capsulatus* and had a redox potential of -390 mV rather than +290 mV as seen in the wild-type enzyme. The mutation resulted in a loss of photosynthetic activity in the bacterium, suggesting that the methionine ligand to the heme is essential for the reduction of cytochrome c1 by the iron sulphur centre (250).

![Figure 5.1 cytochrome c1 of the cytochrome bc1 complex](image)

A second example of a heme-containing protein with methionine axial heme ligation is bacterioferritin. The ferritins are a family of iron-storage proteins, preventing iron
from reacting with dioxygen and forming radical species which are damaging to the cell (252). Bacterioferritins are a subfamily of ferritins which are found in bacteria and contain a heme b. They are large proteins with 24 subunits each of ~ 18 KDa, which together are capable of binding up to 4000 iron atoms (253). In addition to the heme group they contain a di-iron centre which is the site of oxidation of ferrous iron by molecular oxygen. This is a necessary step for iron uptake (254). The heme contained within bacterioferritin is a heme b with bis methionine heme iron ligation (255,256). Andrews et al. mutated the axial methionine residues to leucine or histidine in E. coli bacterioferritin and found that the enzyme no longer contained heme, but was still correctly assembled and capable of iron uptake (257). The role of the heme group in bacterioferritin is not known, although it has been suggested that it has a role in iron release and mediates the potential of the iron stored in the bacterioferritin (258).

Figure 5.2 Bacterioferritin heme and axial methionine residues

The heme b group of bacterioferritin can be seen in red with the two axial methionines seen above and below the heme group. The heme is located between 2 polypeptides and the axial ligands are M52 from each polypeptide (259). Image generated using the structural coordinates in the PDB file 1BFR. Image generated using Pymol.

Another example of a methionine-ligated heme protein is cytochrome b_{562}, which is a small, simple electron transfer cytochrome from the E. coli periplasm, composed of 4 \( \alpha \)-helices totalling only 12 KDa and with His/Met heme iron ligation (260). A mutant version with bis-methionine ligated heme has been generated and characterised by
Chapter 5 Characterisation of A264C/M P450 BM3 mutants

Barker et al (261,262). They found that the H102M mutant did form bis-Met heme iron ligation, but not entirely in the ferric enzyme. However, the bis-Met ligation became stabilised in the ferrous form.

A number of studies have also been carried out on 5-coordinate cytochromes where sulphur donor ligands have been used as 6th ligand. Sono et al. carried out one such study on cytochrome P450 cam and myoglobin (166). They showed that it is possible to form thiolate complexes to myoglobin, which contains histidine as proximal ligand, using the thiolate analogues p-chlorothiophenol and 1-propanethiol. They also formed a thiolate complex to the distal position of P450 cam, but this was not stabilised in the thiolate form and could be shifted to thiol ligation with varying pH. Sono et al. also successfully used dimethyl sulphide as a thioether analogue to bind to P450 cam. These mutants were characterised spectroscopically, but no thermodynamic analysis was done to assess the effects on the redox potentials of the new heme centres (166).

In Chapter 3 and Chapter 4, mutations to the A264 position of P450 BM3 were made which resulted in novel heme ligation states. The only information available to date on bis-Cys or Cys/Met ligated heme proteins used exogenous cysteine and methionine analogues, rather than employing mutagenesis successfully to enable both 5th and 6th sulphur-containing ligands to come from the polypeptide itself. It is therefore of interest to create A264C and A264M mutants of P450 BM3 with the aim of producing novel cytochrome P450 variants with bis-Cys or Cys/Met axial heme ligation. The chapter reports on the mutagenesis to generate the heme domain and flavocytochrome A264M and A264C mutants, the expression and production of the proteins and their subsequent spectroscopic, kinetic, thermodynamic and structural characterisation.
Chapter 5 Characterisation of A264C/M P450 BM3 mutants

5.2 Results

5.2.1 Mutagenesis

The A264C and A264M mutants were successfully generated in both heme domain and intact flavocytochrome P450 BM3 in the plasmids pBM20 and pBM25, respectively, as described in the Methods section. Following the mutagenic PCR reaction and digestion with the restriction endonuclease DpnI to remove non-mutated template DNA, 5 μl of the PCR reaction mixtures were run on an ethidium bromide-stained 0.8 % agarose gel in order to verify the presence of the mutation. The gel used to verify the successful PCR reaction to generate the A264M heme domain mutant is shown in Figure 5.3.

![Ethidium bromide-stained 0.8 % agarose gel of A264M heme domain PCR product](image)

Following the successful PCR reactions, transformation into Novablue™ competent cells, and growth on LB plates containing ampicillin, 6 colonies were picked for each mutant and plasmids prepared from the derived cultures as described in the Methods section. The mutagenic primer used to generate the A264M mutant also contained silent mutations which introduced an additional BspHI restriction enzyme site, as described in Table 2.5 of the Methods section. Digestion with BspHI therefore verified the success of the mutagenesis. This digest is shown in Figure 5.4.

Figure 5.3 Ethidium bromide-stained 0.8 % agarose gel of A264M heme domain PCR product

5 μl of DpnI digested A264M heme domain mutagenic PCR product is shown in lane 2 against 1 Kb NEB DNA marker shown in lane 1. The marker band indicated at 3 Kb contains ~ 50 ng of DNA.
The mutagenic primers used to generate the A264C mutation did not contain the BspHI restriction enzyme site. The mutagenic primers were initially designed with the BspHI site present, but the PCR reaction was unsuccessful, even after conditions were changed to attempt to optimise the PCR reaction. In order to generate the A264C mutant, mutagenic primers were therefore redesigned without the silent mutations to generate the BspHI restriction enzyme site, reducing the number of mismatches, therefore increasing the probability that the primer would anneal to the backbone DNA. Redesigning the mutagenic primers was successful and the DNA was amplified under conditions described in Table 2.1 of the Methods section.

The A264M and A264C mutants were generated in the intact flavocytochrome DNA construct in the same manner as the heme domain constructs.

DNA was fully sequenced by the dideoxy chain termination method (PNACL facility, University of Leicester) to check for the presence of desired mutations, and ensure that no unwanted secondary mutations were present. Figure 5.5 shows the chromatogram data for the mutated region from the A264M heme domain sequencing. Primers used for the sequencing of both intact flavocytochrome constructs and heme domain constructs are detailed in Table 2.11 of the Methods section.
Chapter 5 Characterisation of A264C/M P450 BM3 mutants

Figure 5.5 Chromatogram data for A264M heme domain sequencing
The A264M residue, highlighted with a red box surrounding, now has the methionine codon ATG. The oligonucleotide used for the generation of the A264M mutation is underlined in red. The sequence corresponds entirely to the wild-type sequence with the exception of desired mutations.

5.2.2 Overexpression and purification of A264M/C mutants

Both heme domain and intact flavocytochromeP450 BM3 in wild-type and mutant forms were overexpressed and purified as described in the Methods section. The purity of all enzyme preparations was verified by SDS-PAGE gel analysis prior to use of enzymes for structural or biophysical analysis. A SDS-PAGE gel containing pure A264M heme domain is shown in Figure 5.6. All mutants and wild-type enzymes were fractionated to the same level of purity, with no changes to protein stability (i.e. levels of proteolysis) observed.
Chapter 5 Characterisation of A264CM P450 BM3 mutants

Figure 5.6 10 % SDS-PAGE gel showing pure P450 BM3 A264M heme domain
Shown in lane 1 is purified A264M heme domain and in lane 2 NEB broad range pre-stained protein marker, with the sizes indicated. The A264M heme domain is a single band of pure protein corresponding to the expected size of 54 KDa.

5.2.3 Calculation of heme concentration
Neither the A264M or A264C mutants formed a full or stable reduced carbon monoxide adduct at 450 nm. Therefore, the protein concentration could not be calculated accurately by the method of Omura and Sato (31). The inability of the A264M and A264C mutants to complex fully with carbon monoxide following reduction with dithionite will be discussed further in subsequent sections of this chapter. The concentration of all heme domain and flavocytochrome wild-type and mutant constructs were therefore calculated by the method of Berry and Trumpower (190), with the assumption made that the heme to protein ratio was 1:1. Shown in Figure 5.7 is the pyridine hemochromagen spectrum used for the calculation of the A264M heme domain concentration.
Chapter 5 Characterisation of A264C/M P450 BM3 mutants

Figure 5.7 Pyridine hemochromagen spectra of A264M and A264C heme domains
Shown red is the A264M mutant and in green the A264C mutant. Both mutants have maxima in the Q-band at 556 nm, which is indicative of heme b. The protein concentrations were calculated as 6.5 µM and 5.1 µM for the shown samples of A264M and A264C respectively using the extinction coefficient of 24 M⁻¹cm⁻¹ for the absorption difference between 550 nm and 535 nm in the spectrum (A₅₅₀ - A₅₃₅).

5.2.4 Comparison of wild-type and A264M/C mutant UV-visible spectra

UV-visible spectra of oxidised, substrate-free forms of wild-type, A264M and A264C heme domains are shown below in Figure 5.8.
Chapter 5 Characterisation of A264C/M P450 BM3 mutants

Figure 5.8 Oxidised A264C, A264M and wild-type heme domain spectra
The wild-type spectrum is shown in green, the A264C mutant in red and the A264M mutant in blue. Approximately 2.5 μM protein was used to collect each spectrum. The wild-type spectrum has its Soret maximum at 418 nm, the A264C and A264M mutants have Soret maxima at 418 nm and 416 nm, respectively. Wild-type and A264C have their α-band at 569 nm and β-band at 535 nm, the A264M mutant has its α-band at 566 nm and β-band at 534 nm.

Table 5.1 UV-visible spectral maxima of wild-type and A264C/M mutant P450 BM3

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<thead>
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<th></th>
<th>Soret (nm)</th>
<th>α-band (nm)</th>
<th>β-band (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>418</td>
<td>569</td>
<td>535</td>
</tr>
<tr>
<td>A264C</td>
<td>418</td>
<td>569</td>
<td>535</td>
</tr>
<tr>
<td>A264M</td>
<td>416</td>
<td>566</td>
<td>534</td>
</tr>
</tbody>
</table>

Comparison between the oxidised A264M/C mutants and the wild-type heme domain spectra reveal a number of changes. Both the A264C and A264M mutants have a shoulder in their Soret band at ~ 390 nm, indicating that a proportion of each mutant is purified in the high spin form, unlike the wild-type spectrum, which is typical of low spin, ferric P450 with cysteine/water coordination to the heme iron. Aside from the
high spin content, the A264C mutant shows no spectral differences to the wild-type enzyme, suggesting that the iron coordination is not changed from the wild-type. The shift in Soret maximum from 418 nm in the wild-type enzyme to 416 nm in the A264M mutant, and slight decrease in the intensity of the α-band suggest that the M264 residue affects the heme iron, possibly through the formation of a novel methionine/cysteine ligation to the heme, as was shown to occur between the E264 mutant residue as discussed in Chapter 3 and the K/H/Q264 mutant residues as discussed in Chapter 4.

5.2.5 Substrate binding

Binding of the fatty-acid substrates arachidonic acid, palmitoleic acid, palmitic acid, myristic acid and lauric acid was analysed for the wild-type, A264M and A264C heme domains. As was discussed in Chapter 3, the wild-type enzyme heme iron shifts from low- to high-spin on binding of substrate, with a concomitant shift in the Soret maximum from 418 nm to 390 nm. The spectral changes on binding of arachidonic acid to each of the mutants are shown below in Figure 5.9 and Figure 5.10.
Chapter 5 Characterisation of A264C/M P450 BM3 mutants

Absorbance

0.30
0.25
0.20
0.15
0.10
0.05
0.00
20 30 40 50 60 70
Wavelength (nm)

0.12
0.08
0.04
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.15
0.20
0.25
0.30

[Arachidonate] (µM)

Figure 5.9 Binding of arachidonic acid to A264C heme domain.
Arachidonic acid was titrated from a 33 mM stock in 0.1 µl aliquots against ~ 2.5 µM A264M heme domain. The red spectrum is the starting spectrum, collected prior to the addition of any arachidonic acid and the blue spectrum was the final spectrum collected and contains 49.5 µM arachidonic acid. Black spectra show intermediate points in the binding titration. Shown in inset are the data points generated by plotting the maximal absorbance changes induced ($\Delta A_{388} - \Delta A_{421}$) against the relevant arachidonic acid concentration. The data were fitted to a rectangular hyperbola function (Equation 2) generating a $K_d$ of $6.37 \pm 0.33$ µM.
Chapter 5 Characterisation of A264C/M P450 BM3 mutants

Figure 5.10 Binding of the substrate arachidonic acid to A264M BM3 heme domain

Shown in red is the starting spectrum, collected prior to the addition of any arachidonate. The blue spectrum shows the final spectrum collected and corresponds to an arachidonate concentration of 9.6 μM. Spectra shown in black are a number of those collected following each addition of arachidonate, which was added in 0.1 μl aliquots from a 33 mM stock, in the solvent ethanol. Plotting the maximal absorbance change induced (ΔA_{388} - ΔA_{421}) against the relevant concentration of arachidonic acid generated the data shown in inset. The data were fitted to a rectangular hyperbola (Equation 2) generating a $K_d$ of 0.35 ± 0.02 μM.

Comparison between the binding of arachidonic acid to the wild-type enzyme, (Shown in Figure 3.10 of Chapter 3) and to the A264C and A264M mutants, shown in Figure 5.9 and Figure 5.10 respectively, demonstrates that the mutants both show a typical spectral shift from the low-spin form to the high-spin form with absorbance increasing at 390 nm. The mutants form a significantly smaller proportion of high-spin complex at saturating concentrations of arachidonic acid compared to the wild-type enzyme, with the A264C mutant only forming around 25 % high-spin and the A264M mutant forming around 60 %, with the wild-type enzyme capable of giving over 80 % spin shift to the high-spin form in the same arachidonic acid concentration range. The lower proportion of the A264C/M mutants which shift to the high-spin form on
saturation with substrate suggests that the mutations are in some way affecting the enzyme's ability to bind substrate or the ability of the heme iron to undergo a spin state shift to the high-spin form. This could be a result of a number of factors, but at this stage no solid conclusion can be made. It is possible that the enzyme is in a heterogeneous mix, where some of each mutant contains cysteine/cysteine or cysteine/methionine ligation to the heme iron. However, it is also possible that the mutations are affecting the enzyme without the new amino acid side chains directly ligating to the heme iron, but instead inhibiting the binding of substrate, possibly by providing steric hindrance to substrate access to the heme environment. Shown below in Table 5.2 are the dissociation constants calculated for each of the mutants and the wild-type enzyme from the optical binding assays using the A264M/C heme domains and a variety of long chain fatty acids.

### Table 5.2 Binding constants calculated for substrate binding to wild-type, A264C and A264M BM3 heme domains

Each substrate was titrated against approximately 2.5 μM of the relevant enzyme until no further optical change was observed. All absorption change versus substrate concentration data were fitted to a rectangular hyperbola (Equation 2), as described in the Methods.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Wild-type</th>
<th>A264M</th>
<th>A264C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonate</td>
<td>0.55 ± 0.05</td>
<td>0.35 ± 0.02</td>
<td>6.4 ± 0.3</td>
</tr>
<tr>
<td>Palmitoleate</td>
<td>3.5 ± 0.2</td>
<td>0.14 ± 0.05</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Palmitate</td>
<td>11.3 ± 0.4</td>
<td>7.4 ± 0.4</td>
<td>ND*</td>
</tr>
<tr>
<td>Myristate</td>
<td>6.9 ± 0.4</td>
<td>0.89 ± 0.31</td>
<td>ND*</td>
</tr>
<tr>
<td>Laurate</td>
<td>89 ± 15</td>
<td>17.8 ± 0.9</td>
<td>ND*</td>
</tr>
</tbody>
</table>

No spectral shifts on addition of substrate were observed.

Comparison between the binding spectra obtained for each of the mutants with those generated for the wild-type enzyme show that the optical changes seen on arachidonic acid binding (as shown in Figure 5.9 and Figure 5.10) are similar with each of the substrates tested, although the extent of absolute spin state change induced varies between individual substrates. Binding of all of the fatty acids tested has become tighter in the case of the A264M mutant, but a smaller overall shift to the high-spin
form was seen in each case. However the A264C mutant shows significantly larger
dissociation constants, again with a smaller amount of enzyme converted to the high-
spin form for arachidonate and palmitoleate. In the cases of palmitate and the shorter
chain saturated fatty-acids, no significant spectral shift was seen for the A264C mutant
in the concentration range tested (i.e. within the solubility ranges achievable for these
fatty acids), consistent with their binding also being much weaker to the A264C heme
domain than to wild-type heme domain.

5.2.6 Inhibitor binding
As has been discussed previously, a number of molecules act as inhibitors to the
cytochromes P450 by forming a strong, direct ligation to the heme iron thus preventing
the binding of oxygen to the heme iron and any enzyme turnover. The inhibitors 4-
phenylimidazole and sodium cyanide were titrated against the wild-type and A264C/M
mutants in order to assess any changes in the affinities for the inhibitors between the
wild-type and mutant enzymes. As was shown in Figure 3.14 (Chapter 3) binding of
4-phenylimidazole to wild-type BM3 causes the Soret maximum to shift from 418 nm
to 424 nm on saturation with 4-phenylimidazole, with a concomitant increase in the
intensity of the α-band and a decrease in the intensity of the β-band.
Chapter 5 Characterisation of A264C/M P450 BM3 mutants

Figure 5.11 4-Phenylimidazole binding to A264C
0.1 μl aliquots of 500 mM 4-Phenylimidazole stock (solubilised in assay buffer) were titrated against ~ 2.5 μM A264C BM3 heme domain. The starting spectrum, which contains no 4-phenylimidazole, is shown in red and the final spectrum, which contains 120 μM, is shown in blue. Shown in inset are the data points generated by plotting the maximal absorbance change (ΔA_{435} minus ΔA_{415}) against the corresponding concentration of 4-phenylimidazole. The data were fitted to a rectangular hyperbola (Equation 2), generating a $K_d$ of 52 ± 2.8 μM.
Both the A264M and A264C mutants have shown spectral changes on the binding of 4-phenylimidazole that are very similar to the changes observed for the wild-type enzyme. Binding of 4-phenylimidazole to the mutants caused a shift in Soret maximum to 420 nm in the A264M mutant and to 422 nm in the A264C mutant, rather than to 424 nm as seen in the wild-type enzyme. 4-Phenylimidazole binds to the wild-type enzyme with a $K_d$ of 5.8 ± 1.0 μM, significantly tighter than it binds to either the A264C or A264M mutants, which gave $K_d$ values of 52 ± 2.8 μM and 444 ± 44 μM respectively. Full conversion to the 4-phenylimidazole bound form is not seen in either mutant.
These results show that the affinity for 4-phenylimidazole is significantly reduced in both the mutants and confirm results seen in the substrate binding titrations, which showed that the mutations impacted on substrate affinity as measured by optical titrations. Potentially, the apparent change in affinity for 4-phenylimidazole is caused by either new ligation states formed between the M/C264 residue side chains and the heme iron (and the requirement of high concentrations of the exogenous azole in order to displace the endogenous ligand), or possibly through other effects the mutation is having on the enzyme (e.g. structural or electronic effects on the heme and its environment) which weaken the binding of 4-phenylimidazole.

Figure 5.13 Sodium cyanide binding to A264C heme domain
Sodium cyanide was titrated against ~ 2.8 μM A264C heme domain in 0.2 μl aliquots from a 1 M stock. Shown in red is the starting spectrum, collected prior to the addition of any sodium cyanide, the blue spectrum shows the final one collected, which contains 8.25 mM sodium cyanide. The spectra shown in black are a number of those collected during the titration. Shown in inset are data points generated by creating difference spectra and plotting the maximal absorbance change against the corresponding concentration of sodium cyanide (ΔA_{446} minus ΔA_{417}). The data were fitted to the Hill function generating a half saturation concentration for the ligand of 4.99 ± 0.54 mM, with a corresponding Hill coefficient of 2.38 ± 0.28.
Binding of sodium cyanide to A264M BM3 heme domain

Shown in red is the spectrum prior to the addition of any sodium cyanide. The sodium cyanide was added as 0.2 μl aliquots from a 1 M stock solution and the blue spectrum is that of the final spectrum collected following the addition of 12 mM sodium cyanide, the black spectra show a number of intermediate concentrations of sodium cyanide. Data were analysed by generating difference spectra and plotting the maximal absorbance change induced against the corresponding concentration of sodium cyanide (ΔA_{445} minus ΔA_{405}). The data were fitted to the Hill function, generating a half saturation NaCN concentration of 4.46 ± 0.06 mM and a Hill coefficient of 2.76 ± 0.09.

Comparison between binding of sodium cyanide to wild-type BM3, as shown in Figure 3.18 (Chapter 3), and to the A264C/M mutants reveals that similar spectral shifts occur as the cyanide coordinates to the P450 heme iron. On binding of sodium cyanide the wild-type enzyme Soret maximum shifts from 418 nm to 445 nm. The same general spectral shift is observed for the A264C and A264M mutants. However, both mutants do not show complete conversion to the new form with Soret maximum at 445 nm, instead having split species with a proportion of the enzyme remaining in its original spectral form. A single isosbestic point in the Soret region in both cases indicates that there is an apparent simple transition between the two species. The $K_d$ data in each case was computed by plotting the maximal absorbance change induced...
on cyanide binding against the corresponding concentration of sodium cyanide. In the case of the wild-type enzyme the data described a rectangular hyperbola, and the fit to these data generated a $K_d$ of $1.68 \pm 0.68$ mM. Both the A264M and A264C optical change data did not describe rectangular hyperbolae. In both cases the plots of $\Delta$Abs versus [cyanide] were sigmoidal. The data were instead fitted to the Hill function. Both mutants showed saturation in their data fit, therefore the apparent incomplete conversion to a Soret maximum at 445 nm is not due to non-saturation with sodium cyanide, and an end point in the binding titration has been reached. The Hill function describes cooperative binding, and produced a fit with low errors for cyanide binding to both mutants with a $h$ value greater than one indicating positive cooperativity. As with the A264Q mutant (Chapter 4) the sigmoidal data could be due to cooperativity in the binding of sodium cyanide to the mutants, and/or due to conformational rearrangement on binding of sodium cyanide. It is also possible that the enzymes are in multiple conformations, each of which binds sodium cyanide with different affinities. There are isosbestic points present at 431 nm in the binding of sodium cyanide to each mutant, suggesting that there are only two species predominant in the solution at the end of the titration, namely one with cyanide ligated to the heme iron and one without. Again, as with the A264Q mutant, the incomplete conversion to the cyanide ligated form may be indicative of different forms of the mutant, possible with different heme distal ligations, which have differing affinities for sodium cyanide — i.e. cyanide is likely to bind more weakly to any form with an endogenous amino acid ligand to the heme iron, due to the requirement for displacement of this ligand.

5.2.7 Redox Potentiometry

Redox potentials were determined for the mutant and wild-type heme domains in both substrate-free and substrate-bound forms. Wild-type data are shown, and discussed in Figure 3.19 and Figure 3.21 of Chapter 3. A264C and A264M mutant redox potentials are shown below.
Figure 5.15  Redox titration of substrate-free A264C heme domain

Shown in red in the main figure is the fully oxidised spectrum for the A264C domain (~9 μM), which corresponds to a potential of -101 mV, shown in blue is the most reduced spectrum generated, with a potential of -464 mV. Shown inset is the plot of absorbance at 390 nm against the corresponding potential. The data were fitted to a 1 electron Nernst equation, yielding a redox potential of -385 ± 5 mV.

Redox potentials were also measured for both the A264C and A264C mutants following apparent oxidation with formate and H2. The resultant redox potentials are shown in Figure 5.17 and Figure 5.18 and detailed in Table 5.3, and discussed below.
Redox titration of substrate-free A264M heme domain

Shown in red is the oxidised spectrum of A264M heme domain (~10 μM) collected prior to the addition of any sodium dithionite reductant, with its Soret maximum at 416 nm. The spectrum shown in green was the final spectrum collected, following addition of the reductant sodium dithionite until such point that no further absorption change was induced. The full reduced spectrum has its Soret maximum at 413 nm and a shoulder at ~440 nm. The black spectra were collected at sequential stages during the reduction process. Shown in inset is the absorbance at 437 nm at the various points in the titration, which was plotted against the corresponding potential. The data were fitted to the one electron Nernst equation generating a redox potential of -220 ± 8 mV.

Redox potentials were also measured for both the A264M and A264C mutants following apparent saturation with arachidonic acid. The resultant redox potentials are shown in Figure 5.17 and Figure 5.18 and detailed in Table 5.3, and discussed below.
Figure 5.17 Redox titration of substrate-bound A264C heme domain

Shown in red is the oxidised spectrum of arachidonate-bound A264C heme domain (~ 7 μM) collected prior to the addition of any dithionite with its Soret maximum at 418 nm and 393 nm. Shown in blue is the final reduction spectrum collected following the addition of dithionite until no further spectral changes were observed, with its Soret maximum at 410 nm. The spectra shown in black were collected at sequential stages through the reduction process. Shown in inset is the absorbance at 552 nm at each stage of the reduction plotted against the corresponding potential. The data were fitted to a one electron Nernst equation generating a potential of -240 ± 6 mV.
Chapter 5 Characterisation of A264C/M P450 BM3 mutants

Figure 5.18 Redox titration of substrate-bound A264M heme domain
Shown in red is the oxidised spectrum of arachidonate-bound A264M heme domain (~9 μM) collected prior to the addition of any sodium dithionite reductant, with its Soret maximum at 398 nm. The spectrum shown in blue was the final spectrum collected, following addition of the reductant sodium dithionite until such point that no further absorption change was induced. The full reduced spectrum has its Soret maximum at 410 nm and a shoulder at ~ 435 nm. The black spectra were collected at sequential stages during the reduction process. Shown in inset is the absorbance at 398 nm at the various points in the titration, which was plotted against the corresponding potential. The data were fitted to the one electron Nernst equation generating a redox potential of -227 ± 7 mV.

Table 5.3 Mutant and wild-type heme reduction potentials (mV)
Redox potentials for mutant and wild-type heme domains were calculated in both the absence of substrate and in the presence of an apparently saturating concentration of the substrate arachidonic acid. Redox potential data are cited with reference to the standard hydrogen electrode (SHE).

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>A264C</th>
<th>A264M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate-free</td>
<td>-391</td>
<td>-385 ± 5</td>
<td>-220 ± 8</td>
</tr>
<tr>
<td>+ Arachidonate</td>
<td>-283</td>
<td>-240 ± 6</td>
<td>-227 ± 7</td>
</tr>
</tbody>
</table>
Comparing the reduction spectra of the A264C mutant and the wild-type heme domain, as shown in Figure 3.19 Chapter 3, it can be seen that in the substrate-free form the mutant displays typical reduction spectra, with fusion of the α and β bands to form an apparent single band with a maximum at 550 nm, and with the Soret maximum shifting towards 410 nm, indicative for the shift from ferric to ferrous thiolate-ligated heme iron. The redox potentials obtained for the substrate-free A264C mutant are similar to those obtained for the wild-type enzyme. As was shown in Figure 5.9, saturation with arachidonic acid does not induce a complete conversion to the high-spin form, with only ~65% of the enzyme shifting to the high-spin form. The A264C mutant shows a positive shift in redox potential of ~140 mV on saturation with the fatty-acid. In earlier work it was shown that the wild-type enzyme also shows a positive shift in potential of ~100 mV. Previous studies have also indicated that the wild-type enzyme also undergoes a shift in heme iron redox potential of 130-140 mV on substrate saturation (100). The redox titration data presented here for the wild-type and A264C mutant indicate that a similar elevation of heme iron reduction potential is observed on substrate binding to both enzymes, despite the apparent lower conversion of the mutant enzyme to the high-spin form on substrate binding. A possibility is that a “substrate-bound”-like conformational state is adopted by fatty acid-saturated A264C, regardless of the extent of conversion to high-spin heme iron, and that it is this conformer that has altered redox properties.

Comparison between the reduction of the A264M mutant and the wild-type enzyme shows a number of marked differences. On reduction, the Soret maximum does not entirely shift to 410 nm, as it does in the wild-type enzyme, but instead splits with one species at 413 nm and a shoulder at 437 nm. Comparison between these data, the reduction of the A264K and A264H mutants (Figure 4.17 and Figure 4.18 Chapter 3) and work published by Perera et al. (164) suggests that the proximal cysteine is in the protonated thiol form, rather than the thiolate form seen in the wild-type enzyme, for the reduced A264M mutant. On addition of saturating arachidonic acid, optical changes indicate that the A264M mutant shifts to ~60% high-spin. Reduction of this form with dithionite gives a far larger species at 437 nm than is observed for the substrate-free enzyme. In the substrate-free redox spectra a small absorption...
Chapter 5 Characterisation of A264C/M P450 BM3 mutants

maximum can be seen ~ 650 nm and a shoulder at 390 nm, suggesting that there is a small amount of high-content in the substrate-free sample. It is therefore possible that the proximal cysteinate residue of the substrate-bound portion of the enzyme becomes protonated on reduction. It is possible that the shift from low- to high-spin iron with the M264 residue nearby affects the heme such that, on introduction of an electron, the cysteinate becomes protonated (i.e. through electronic or structural influences), or that the binding of substrate affects the position or ligation of the M264 residue such that it destabilises the thiolate ligation, again causing it to become protonated on reduction of the heme iron.

5.2.8 EPR

EPR spectra were collected for each of the P450 BM3 A264 mutants generated, wild-type P450 BM3 EPR spectra were also collected and are shown in Figure 3.23 of Chapter 3 and discussed therein. Spectra were collected by Dr Harriet Seward (University of Leicester) using facilities at the Department of Chemistry, University of East Anglia.
Figure 5.19  EPR spectra for substrate-free and arachidonate-bound forms of A264C, A264M and A264M heme domains

EPR spectra were all collected at 9.668 GHz, 2 mW and 10.8 K with ~500 μM enzyme. Shown in red is the A264C mutant, in green the A264M mutant and in blue the A264M mutant following the addition of arachidonate 500 μM. The g-values for high- and low-spin forms of the hemes are indicated on the spectra in the relevant colour.

<table>
<thead>
<tr>
<th>Wild-type, A264M and A264C g-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
</tr>
<tr>
<td>Wild-type + Substrate</td>
</tr>
<tr>
<td>A264M</td>
</tr>
<tr>
<td>A264M + Substrate</td>
</tr>
<tr>
<td>A264C</td>
</tr>
</tbody>
</table>

Both the A264M and A264C EPR spectra show the presence of a number of distinct EPR signals, suggesting that the heme population is in a mixture of different ligand-
Chapter 5 Characterisation of A264C/M P450 BM3 mutants

bound forms. In the substrate-free, low-spin form the wild-type enzyme has g-values of 2.42 (g_z), 2.26 (g_y) and 1.92 (g_x). Both mutants have species with the same g-values as the low-spin substrate-free wild-type enzyme, therefore a portion of both A264M and A264C have water and thiolate axial ligands. The species seen at 7.991, 3.610 and 1.699 in the A264M mutant can be attributed to high spin, cysteinate ligated P450. Sono et al. studied the binding of sulphur donor ligands to P450 cam, by binding small sulphur-containing compounds to the enzyme and noting changes in UV-visible, EPR and MCD spectra (166). Using dimethylsulfide binding to study thioether complexes they observed shifts in EPR g-values to 2.5 (g_z), 2.27 (g_y) and 1.89 (g_x), from 2.44 (g_z), 2.25 (g_y), and 1.91 (g_x) in the wild-type form with the distal water present as 6th ligand. The changes seen between thioether and water as distal ligand in P450 cam concur with those seen in a proportion of the A264M mutant (namely 2.527 (g_z), 2.25 (g_y) and 1.89 (g_x)), suggesting that a proportion of the enzyme contains the thioether methionine ligand to the heme iron in addition to the proximal cysteinate ligation. The other g_z features seen at 2.386 and 2.428 are possibly also due to the formation of a M264 thioether bond in the distal position on the heme iron, although in this case being influenced by other amino acids around M264 in the active site.

The A264C mutant also shows altered EPR spectra and hence heme iron ligation states when compared with the wild-type enzyme. Comparison between the spectrum obtained for the A264C mutant and the data published by Sono et al. suggest that, as with the A264M mutant spectra, there are interactions between the heme iron and the C264 residue. A proportion of the enzyme is seen in the high-spin form, with g_z = 7.016 and g_y = 4.910 and no detectable g_x signal. This is a less rhombic EPR signal than the wild-type enzyme or the high-spin A264M mutant, suggesting that there have been changes in the iron coordination, possibly with changes to the thiolate bond in relation to the porphyrin plane. As with the A264M mutant, a number of different low-spin species are seen in the EPR spectrum. Comparison between these data and those obtained by Sono et al. (166) suggests that the A264C mutant binds heme iron in a mixture of different ligation states. The signals at 2.423 (g_z) and 1.918 (g_x) are comparable to wild-type low-spin signals and can therefore be attributed to low-spin, thiolate-ligated heme iron with water as distal ligand. The g_z signal seen at 2.372 is comparable to P450 cam with deprotonated dithiothreitol as distal ligand (166), suggesting that a proportion of the A264C mutant has bis-thiolate ligation. A further
species is also seen with $g_z$ at 2.471, comparable to the data set obtained by Sono et al. (263) on binding of exogenous ligands to the thiolate-ligated heme $b$ enzyme chloroperoxidase. Similar EPR spectra were observed when methanethiol was bound to the distal position of chloroperoxidase, and similar spectra were again obtained on binding of propanethiol to P450 cam (166), suggesting that the P450 BM3 species with $g_z$ at 2.471 is due to thiol/thiolate ligation.

EPR data obtained for both the A264M and A264C mutants suggests that, in a proportion of the enzymes, novel ligation is seen with the mutant methionine or cysteine residues ligating to the heme iron, with both thiol and thiolate ligation from the proximal cysteine residue likely in the A264C variant. Comparison between these data and the UV-visible data shown and discussed in section 5.2.4 suggests a discrepancy in the case of the A264C mutant. In the study carried out by Sono et al. (166) binding of the thiolate compounds $p$-chlorothiophenol to P450 cam resulted in split Soret peaks with maxima at 380 nm and 460 nm, it is therefore likely that the species seen in the EPR spectra which have been attributed to thiolate/thiolate A264C BM3 are an artefact of the low temperature required to obtain the EPR spectrum and therefore not seen in the UV-visible spectra obtained at ambient temperature.

**5.2.9 MCD**

MCD spectra were collected in both the near infrared and near-UV-visible regions by Dr Harriet Seward (University of Leicester) using facilities at the Department of Chemistry, University of East Anglia.
Chapter 5 Characterisation of A264C/M P450 BM3 mutants

Figure 5.20 Near-UV-visible region MCD spectra of A264C and A264M P450 BM3 mutants
Shown in red is the A264C mutant and in blue the A264M mutant (both as heme domains). All spectra were collected with protein at a concentration of ~ 500 μM. In the region 450 nm to 900 nm the spectra have been multiplied by 5 to display their features more readily.

As with the A264E/K/H/Q mutants discussed in Chapter 3 and 4, the overall spectral pattern of the A264C and A264M mutants is generally indicative of low-spin, ferric heme. There is some high-spin content visible in the A264M spectrum, observable by the troughs at 396 nm, 573 nm and 656 nm, with a smaller proportion of high-spin heme iron signal visible in the A264C spectrum. The spectrum obtained for the A264C mutant is very similar to the wild-type spectrum shown in Figure 3.26 of Chapter 3. Comparison with MCD spectra of P450 cam with thiol and thiolate as distal ligand obtained by Sono et al. (166) suggest that the A264C mutant contains water as distal ligand and the C264 residue has not ligated to the heme iron at ambient temperature conditions. Comparison between the spectrum obtained for the A264M mutant, wild-type enzyme and P450 cam complexed with thioether substrates does not readily indicate what forms of heme iron ligation occur in the A264M. Thioether-complexed P450 cam shows a general shift in the spectral features to a longer wavelength (by approx. 8 nm), but no significant changes in the relative intensities of
the spectral features. On binding of substrate, wild-type P450 BM3 shows a number of significant spectral changes and also a general shift to longer wavelength in those features which are retained in the high-spin spectrum from the low spin form. It is therefore possible that the A264M mutant contains a proportion of thioether/thiolate ligation, but it is not clearly decipherable from the high-spin content.

**Figure 5.21** Near infrared MCD spectra of A264M and A264C P450 BM3 heme domains

All spectra were collected in a 1 mm pathlength cuvette. Shown in red is the A264C mutant at a concentration of ~ 90 µM with its spectral maximum at 1080 nm, and shown in blue is the A264M mutant at a concentration of ~ 130 µM with its maximum at 1084 nm. The spectrum of substrate-free wild-type heme domain (300 µM) is also shown (green), with its maximum at 1078 nm. With the substrate-bound wild-type P450 BM3 (50 µM, following addition of saturating amounts of arachidonic acid) in cyan. For the substrate-bound wild-type P450 BM3, the spectral maximum is shifted to 834 nm.

The near-infra red MCD spectra of the A264C and A264M mutants concur with the spectral observations made for the near-UV visible MCD spectra discussed above. The A264C mutant spectrum has a low-spin charge transfer band at 1080 nm, only 2 nm shifted from the wild-type enzyme, which has its low-spin charge transfer band at 1078 nm. This small shift suggests that the A264C mutant does not contain bis-
thiolate ligation, as the coordination of a second strongly nucleophilic residue to the heme iron would be expected to produce more significant perturbations of the heme iron. It is possible, however, that a proportion of distal thiol/thiolate ligation does occur at ambient temperature, as the presence of C264 thiol ligation would not significantly perturb the spectrum. The A264C spectrum also has a charge transfer band present at 834 nm which, as in the near UV-visible MCD region, suggests that a small proportion of the enzyme is in the high-spin form. The spectrum of the A264M mutant has its low-spin charge transfer band at 1084 nm, a 4 nm shift from the wild-type enzyme, and contains a significant proportion of high-spin species, with a large charge-transfer band at 834 nm. The shift to 1084 nm suggests that there are some changes in the coordination state of the heme iron, possibly with a proportion of the enzyme now in the thiolate/thioether conformation.

5.2.8 Resonance Raman

Resonance Raman spectra were collected in collaboration with Dr Rachael Littleford (University of Strathclyde). Resonance Raman provides a sensitive method for the analysis of heme proteins, being particularly useful for analysis of e.g. bond deformations and positioning of functional groups in and relative to the horizontal plane of the heme, and also to a lesser extent for analysis of the proximal and distal ligands to the iron, as has been discussed in greater detail in chapter 3. Resonance Raman spectra have been collected and analysed for each of the A264 mutants and for the wild-type heme domains in the low, mid and high frequency ranges.
Chapter 5 Characterisation of A264C/M P450 BM3 mutants

Figure 5.22 Resonance Raman spectra of substrate-free and arachidonate-bound A264C/M and wild-type heme domains of P450 BM3
Shown in black is wild-type P450 BM3, and in red following the addition of arachidonate. In green is the A264C mutant and in blue following the addition of arachidonate. The A264M mutant is shown in cyan and in magenta following the addition of arachidonate. All spectra were collected using ~50 μM enzyme and arachidonate was at a final concentration of 200 μM. The $v_4$ band is indicated.

The $v_4$ band, which is indicative of the oxidation state of the enzyme, is positioned at 1372 cm$^{-1}$ in the wild-type enzyme in both substrate-free and substrate-bound forms, indicating that the heme iron is in the ferric state. The A264M and A264C mutants also have their $v_4$ band at 1372 cm$^{-1}$ in both substrate-free and substrate-bound forms, indicating that they are also in the ferric form. As has been discussed in earlier chapters, the high-frequency region is particularly useful for observing spin-spin state changes (244).
Chapter 5 Characterisation of A264C/M P450 BM3 mutants

Figure 5.23  A264M high frequency resonance Raman spectra
Shown in red is the spectrum collected for the A264M mutant, and in black are the peaks generated by curve fitting, with the residual spectrum shown in blue. Shown in green is the spectrum collected for wild-type P450 BM3. Also indicated are the assignments made based on work by Smith et al. (218).
Figure 5.24  Arachidonate-bound A264M high frequency resonance Raman spectrum
Shown in red is the A264M mutant in the presence of arachidonate (200 μM) and in green the arachidonate-bound wild-type P450 BM3. Curve-fitting for the A264M mutant is shown in black, with the residual data generated by subtraction of the curve fit from the actual spectrum shown in blue. Assignments were made based on the work of Smith et al. (218) and are indicated.
Figure 5.25 A264C high-frequency resonance Raman spectrum
The A264C high-frequency resonance Raman spectrum is shown in red, with the curve fit in black. The residual spectrum, calculated by subtraction of curve fit spectra from the A264C spectrum, is shown in blue- and substrate-free wild-type P450 BM3 heme domain is shown in green. Assignments were made based on work by Smith et al. and are indicated (218).
Figure 5.26 **Substrate-bound A264C high-frequency resonance Raman spectrum**

Shown in red is the spectrum for 50 μM A264C heme domain with 200 μM arachidonate added. Curve fit is shown in black with residual data shown in blue, calculated by subtraction of the curve fit data from the spectrum. Substrate-bound wild-type P450 BM3 heme domain is shown in green. Assignments made based on work by Smith *et al.* (218) are indicated.

Table 5.5 **Principle features of substrate-free and substrate-bound wild type, A264C and A264M resonance Raman**

<table>
<thead>
<tr>
<th></th>
<th>$v_4$ (cm$^{-1}$)</th>
<th>$v_{11}$ (cm$^{-1}$)</th>
<th>$v_3$ (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1372</td>
<td>1565</td>
<td>1500</td>
</tr>
<tr>
<td>Wild type + Substrate</td>
<td>1372</td>
<td>1563</td>
<td>1481/1500</td>
</tr>
<tr>
<td>A264C</td>
<td>1372</td>
<td>1562</td>
<td>1498/1511</td>
</tr>
<tr>
<td>A264C + Substrate</td>
<td>1372</td>
<td>1562</td>
<td>1498/1511</td>
</tr>
<tr>
<td>A264M</td>
<td>1372</td>
<td>1567</td>
<td>1482/1500</td>
</tr>
<tr>
<td>A264M+ Substrate</td>
<td>1372</td>
<td>1567</td>
<td>1482/1500</td>
</tr>
</tbody>
</table>
Chapter 5 Characterisation of A264C/M P450 BM3 mutants

The high-frequency region of resonance Raman spectra of heme-containing enzymes is particularly useful for interpreting changes to the spin state of the heme iron. The $v_3$ bands are found at 1481 cm$^{-1}$ and 1500 cm$^{-1}$ in the wild-type enzyme, with the 1500 cm$^{-1}$ band being dominant in the substrate-free enzyme, and the two bands being of approximately equal intensity in the substrate-bound enzyme. The A264M mutant has its $v_3$ bands at 1482 and 1500 cm$^{-1}$ in both the substrate-free and substrate-bound forms, and the intensities change in the same manner as the wild-type enzyme, showing that the enzyme is shifting to the high-spin form on substrate-binding. The band intensities in the substrate-free form also suggest that a proportion of the enzyme is in the high-spin form prior to the addition of any substrate, possibly due to the enzyme co-purifying with some substrate-bound. The A264C mutant has its $v_3$ bands at 1498 cm$^{-1}$ and 1511 cm$^{-1}$ in both substrate-free and substrate-bound forms, with the same shifts in intensity on substrate-binding as are seen in the wild-type enzyme. The shift in the wavenumber of the bands suggests that the A264C mutation has some effect on the heme iron, possibly on the splitting of the d-orbital energies of the heme iron. Also of importance in the high-frequency resonance Raman region is the $v_{11}$ band, which reports of the in-plane asymmetry of the heme group and is therefore affected by the ligation state of the heme iron. In the wild-type enzyme it decreases slightly in intensity on substrate-binding, as the distal water is displaced by substrate, which reduces the doming of the heme macrocycle. As was discussed in Chapter 4, the A264K and A264H mutants have no discernible $v_{11}$ band as the K/H264 ligations have reduced the asymmetry of the heme such that there is no longer a band present. In the wild-type enzyme the $v_{11}$ band is found at 1565 cm$^{-1}$, and at 1562 cm$^{-1}$ and 1567 cm$^{-1}$ in the A264C and A264M mutants, respectively, in both substrate-free and substrate-bound forms. The relative intensity of the $v_{11}$ band, when compared to the $v_4$ band, is reduced in the A264C mutant on substrate-binding, and is not significantly changed on substrate-binding in the A264M mutant. The relative intensities are, however, larger than in the wild-type enzyme, suggesting that any changes brought about by the mutations are increasing the asymmetry of the heme plane. This is possibly due to an equilibrium where an amount of the enzyme retains wild-type water as 6$^{th}$ ligand or has substrate close to the iron, and an amount of the enzyme has weak interactions between the M/C264 residues and the heme iron which do not force the heme group into a planar conformation.
5.2.9 Steady-state kinetics

The rates of substrate-dependent NADPH oxidation were monitored in each of the mutant flavocytochromes and in the wild-type enzyme to assess how changes in the enzyme brought about by the mutations have affected the kinetic parameters for substrate oxidation.

![Graph showing arachidonate-dependent NADPH oxidation by flavocytochrome P450 BM3 A264M mutant]

**Figure 5.27** Arachidonate-dependent NADPH oxidation by flavocytochrome P450 BM3 A264M mutant

Rates of NADPH oxidation were monitored, at various concentrations of arachidonate. Points shown are the averages from at least 3 determinations at each point. The rates so determined are plotted against the arachidonate concentration. The data have been fitted to the Michaelis-Menten equation, giving a $k_{cat}$ of $115 \pm 4 \text{ min}^{-1}$ and a $K_m$ of $10.3 \pm 1.5 \mu\text{M}$. 
Table 5.6 Rates of substrate-dependent NADPH consumption and associated $K_M$ values for wild-type and mutant flavocytochromes P450 BM3

<table>
<thead>
<tr>
<th></th>
<th>Arachidonate</th>
<th></th>
<th>Laurate</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{\text{cat}}$ (min$^{-1}$)</td>
<td>$K_M$ (µM)</td>
<td>$k_{\text{cat}}/K_M$ (µM$^{-1}$min$^{-1}$)</td>
<td>$k_{\text{cat}}$ (min$^{-1}$)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>16400 ± 185</td>
<td>5.1 ± 0.4</td>
<td>3215</td>
<td>2770 ± 120</td>
</tr>
<tr>
<td>A264C</td>
<td>115 ± 4</td>
<td>10.3 ± 0.4</td>
<td>0.56 ± 0.05</td>
<td>195 ± 5</td>
</tr>
<tr>
<td>A264M</td>
<td>115 ± 4</td>
<td>10.3 ± 0.4</td>
<td>0.56 ± 0.05</td>
<td>195 ± 5</td>
</tr>
</tbody>
</table>

*Apparent linear fit, data in this case are in units of µM$^{-1}$min$^{-1}$.*

As can be seen in Figure 5.27 and Table 5.6, the A264M enzyme is catalytically active. It performs substrate-dependent NADPH oxidation, although at rates significantly lower than those catalysed by the wild-type enzyme. The A264C mutant essentially showed linear dependence of NADPH oxidation rate on fatty acid concentration, rather than the hyperbolic dependence seen with the A264M mutant and wild-type enzyme, with both laurate and arachidonate as substrates. The second order rate constant determined from the linear dependence observed with the A264C mutant is generated from the line of best fit through the rate versus substrate concentration data. Substrate binding shown in section 5.2.5 showed that although the A264C mutant is capable of shifting from low- to high-spin on substrate-binding, it has significantly higher dissociation constants than the wild-type enzyme. The predicted concentrations of substrate required to create a near-saturated complex are beyond their levels of solubility in aqueous solution. Laurate is soluble to ~950 µM in assay buffer and arachidonate apparently up to ~330 µM (with 10 µl of a 33 mM stock solution in the solvent ethanol added to give a final assay volume of 1000 µl at the highest concentration used).
5.2.10 Product formation

Steady-state measurements showed that both the A264C and A264M mutants have significantly high levels of substrate-dependent NADPH oxidation. In order to verify if products were formed during NADPH consumption, or whether all NADPH was consumed in futile turnover of oxygen, analysis of lipid products was performed. Mutant or wild-type flavocytochromes P450 BM3 were incubated with the substrates laurate and NADPH. Remaining substrate and products were then isolated by extraction into dichloromethane as described in the Methods and mass spectra collected. The mass of the substrate laurate is 199 amu, and the product has an additional oxygen atom, giving it a mass of 215 amu. It is also possible to see dihydroxylaurate as product with a mass of 231 amu (264). As was shown in Chapter 3, the wild-type enzyme shows complete conversion of the substrate laurate to monohydroxylated product with a mass of 215 amu. Shown below in Figure 5.28 and Figure 5.29 are positive ionisation mass spectra showing the conversion of laurate to product by the A264M and A264C flavocytochrome P450 BM3 mutants.

Figure 5.28 Mass spectrum of organic phase isolates following incubation of lauric acid with A264C flavocytochrome P450 BM3 and NADPH
A limited amount of product can be seen with a mass of 215 amu, but the main component of the mass spectrum is the substrate, with a mass of 199 amu. A number of other small features are seen on the mass spectrum, these correspond to contaminants present in the commercial lipid substrate, which were also seen in control experiments.
Chapter 5  Characterisation of A264C/M P450 BM3 mutants

Figure 5.29  Mass spectrum of organic phase isolates following incubation of lauric acid with A264M flavocytochrome P450 BM3 and NADPH

The A264M mutant has formed a small amount of product with a mass of 215 amu, but as was seen with the A264C mutant, the largest component of the spectrum is the substrate laurate with a mass of 199 amu.

Comparison between the mass spectra for substrate-turnover by the A264C, A264M and wild-type flavocytochromes P450 BM3 shows that the catalytic efficiency of both mutant enzymes is severely diminished when compared to that of the wild-type. All samples were incubated overnight with substrate and NADPH, allowing sufficient time for all NADPH to be oxidised. Although laurate is a relatively weak binding fatty acid and has slower rates of substrate-dependent NADPH oxidation compared with longer chain substrates such as palmitate and arachidonate, it has higher aqueous solubility compared to other longer chain fatty acids and does not require the addition of organic solvent to facilitate its suspension in aqueous media, making it a better substrate to use in these turnover assays. In addition, it is converted to only a small number of products (often only one) and there are no issues regarding its spontaneous oxidation in solution (as is the case with unsaturated fatty acids such as arachidonate). Full NADPH consumption was verified by collecting UV-visible absorbance spectra following the overnight incubation, which showed that the absorption component from NADPH at 340 nm was completely lost (data not shown).

Although the mutants did not entirely convert substrate to product, they did show some activity, converting approximately 5 % and 30 % for the A264C and A264M mutants to product, respectively.
5.2.11 Uncoupled turnover

The relatively high rates of NADPH oxidation in the absence of substrate and the low quantities of product formed by the mutants, when compared to the wild-type enzyme, suggests that a proportion of the NADPH is being consumed by the enzyme without the formation of oxygenated fatty acid product. The cytochromes P450 have been shown to be capable of uncoupled turnover, forming hydrogen peroxide, superoxide or water in the presence of certain inefficient substrates (157,170). A coupled assay using horseradish peroxidase and o-dianisidine can be used to test for the formation of hydrogen peroxide or superoxide, allowing quantification of the uncoupled turnover levels, as was described in chapter 3. The percentage of NADPH forming hydrogen peroxide or superoxide was calculated for both mutants and the wild-type enzyme, and is show below in Table 5.7.

<table>
<thead>
<tr>
<th></th>
<th>% NADPH forming Hydrogen peroxide</th>
<th>% NADPH forming Superoxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>3.3 %</td>
<td>1.2 %</td>
</tr>
<tr>
<td>Wild-type + laurate</td>
<td>1.7 %</td>
<td>4.1 %</td>
</tr>
<tr>
<td>A264C</td>
<td>1 %</td>
<td>1.5 %</td>
</tr>
<tr>
<td>A264C + laurate</td>
<td>1.4 %</td>
<td>2.4 %</td>
</tr>
<tr>
<td>A264M</td>
<td>20 %</td>
<td>1 %</td>
</tr>
<tr>
<td>A264M + laurate</td>
<td>13 %</td>
<td>1 %</td>
</tr>
</tbody>
</table>

The wild-type, A264C and A264M mutants all apparently show very little uncoupling of NADPH oxidation from fatty acid oxygenation. As was discussed in Chapter 3 and shown in work by Munro et al. (220) the wild-type enzyme produces very little hydrogen peroxide or superoxide, with most reducing equivalents being used for product formation. However, the data above indicate that the A264C/M mutants also produce very little hydrogen peroxide or superoxide in turnover of fatty acids. This
appears counter-intuitive given the much lower levels of oxygenated products observed in reactions with these mutant enzymes. In both mutants, the lipid oxidation assays took significantly longer to reach an end point than the wild-type enzyme, due to their much slower catalytic activities of fatty acid-dependent NADPH oxidation. In addition, assays performed in absence of fatty acid also show very low levels of production of superoxide and peroxide, despite the fact that there is now no possibility of using reducing equivalents for hydroxylation of lauric acid. The apparent low levels of hydrogen peroxide or superoxide formation together with the absence of oxygenated fatty acid product seen in turnover studies in section 5.2.10 is thus confusing and apparently contradictory. The fatty acid-dependent enhancement of NADPH oxidation rate in all cases demonstrates that the process is largely enzyme-catalysed. From assays performed in the presence of fatty acids it might be concluded (for the A264C/M enzymes) that “wasted” electrons from NADP are used in the production of water. However, the fact that in the absence of fatty acids (where there would be expected to be a higher proportion of flavin-dependent oxygen reduction) only similar amounts of peroxide and superoxide are detected suggests that water formation is unlikely to explain the stoichiometric imbalance. Thus, it appears likely that, as with the equivalent experiments carried out with the A264K/H/Q mutants (Chapter 4) radical products (i.e. peroxide and superoxide) of A264C/M turnover may be consumed by reaction with the enzyme, preventing their detection by the end point assay. Potentially, a proportion of the NADPH oxidised could also be used to produce water (which would not be detectable by this assay), but based on comparisons between assays done in the presence and absence of fatty acid this amount is probably quite small. However, it is clear that since the majority of NADPH oxidation is not linked to fatty acid oxygenation in either A264M or A264C enzymes, the non-specific NADPH-dependent reduction of dioxygen to radical products is predominant for these enzymes.

5.2.12 Structural studies

Structural studies were carried out on both the mutants in their heme domain by Dr Helen Toogood (University of Leicester). Crystals were all grown by the sitting drop
technique at 5 °C, initially in a screen based around the conditions used for the A264E mutant and optimised, the final conditions used are described in Table 5.8.

Table 5.8 Crystallisation conditions, resolution and space group of A264M and A264C heme domain mutant crystals

<table>
<thead>
<tr>
<th>Crystallisation conditions</th>
<th>Space group</th>
<th>Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>A264M 100 mM cacodylate pH 6.0, 140 mM MgCl₂, 18% PEG 3350</td>
<td>P₂₁₂₁₂₁</td>
<td>2.5 Å</td>
</tr>
<tr>
<td>A264C 100 mM cacodylate pH 6.0, 100 mM MgCl₂, 18% PEG 3350</td>
<td>P₂₁₂₁₂₁</td>
<td>2.1 Å</td>
</tr>
</tbody>
</table>

Data collection was carried out at the European Synchrotron Radiation Facility (Grenoble, France). Structures were solved via molecular replacement using the structures of wild-type and mutant P450 BM3 heme domain (PDB codes 1SMI, 1FAG, 1JPZ and 1BU7) as search models.

Both the A264M and A264C mutants were found to contain 2 molecules in the asymmetric unit, with each of the 2 molecules the same as each other. The M264 residue coordinates to the heme iron in the A264M mutant structure, while C264 does not coordinate in the A264C structure. The heme region is shown for the A264C and A264M structures in Figure 5.30 and Figure 5.31 respectively.
Figure 5.30 **Heme region of the A264C heme domain crystal structure**
The heme group is shown in stick representation. Shown above the heme is the distal ligated water molecule. Cysteine 400 can be seen below the heme, and Cys 264 above the heme, as can F87 and T260. There is 5.52 Å between the sulphur of C264 and the heme iron, 3.48 Å between the sulphur of C264 and F87, 3.99 Å to T260 and 3.94 Å to the distal water. The distance between the distal water and heme iron is 2.55 Å. Distances measured are indicated as green dashed lines in the image on the left. The image on the right also shows the secondary structure.
Chapter 5 Characterisation of A264C/M P450 BM3 mutants

Figure 5.31 Crystal structure of heme region of A264M heme domain

Shown in the image on the left is the heme, represented as red sticks, with C400 seen below ligating to the heme iron, seen above the heme is M264, which is ligating to the distal position of the heme iron, F87 and T260. There is 4.37 Å between M264 and F87, 5.57 Å between M264 and T260 and 3.81 Å between F87 and T260, with the distances measured indicated as dashed green lines. In the image on the right are the same residues with the secondary structure also shown.

As can be seen in Figure 5.30 C264 does not coordinate to the heme iron, instead sitting against F87, as the Q264 and E264 residues did in their “off” positions in the relevant structures. Cysteine is a shorter residue than all the other mutations made to the 264 position and is possibly too short to reach the heme iron without significant conformational rearrangement. Instead of C264 coordination water is occupying the distal position as is seen in the wild-type enzyme in the absence of substrate. In the A264M mutant structure, shown in Figure 5.31 M264 can be seen forming a thioether bond to the heme iron, with 2.78 Å between the heme iron and M264 sulphur.

In addition to analysing the heme binding region of the A264M and A264C mutants it is also of interest to assess changes to the conformation of the structures in comparison to substrate-free and substrate-bound wild-type P450 BM3. Shown in Figure 5.32 and Figure 5.33 are A264C and A264M mutant structures overlaid with the substrate-free and substrate-bound wild-type structures.
Figure 5.32 Palmitoleic acid bound wild-type P450 BM3 overlaid with A264C/M mutant structures.
Shown in green is the palmitoleic acid bound wild type structure (PDB 1FAG (135)), in blue the A264C mutant and in magenta the A264M mutant. The structures have been overlaid and all show the same conformation. Alignment and image generated using Weblab.
Comparison between Figure 5.32 and Figure 5.33 show clearly that the A264C and A264M mutant are in the substrate-bound conformation rather than the substrate-free conformation. Interestingly, the A264C mutant shows no coordination between C264 and the heme iron and does not have any substrate present in the crystal structure, but is found in the same conformation as the coordinating A264M mutant and substrate-bound wild type structure. This suggests that although the A264C mutation has not resulted in Cys/Cys ligation it is has changed the mutant significantly enough that the crystal form is not in the substrate-free conformation.
5.3 Discussion

This section of work has studied the effects of mutating the A264 residue of P450 BM3 to either of the sulphur containing amino acids cysteine or methionine. Chapters 3 and 4 have shown generation and characterisation of the A264E/K/H/Q mutants. Each of the mutants have some coordination between their mutant 264 residue and the heme iron, generating novel heme coordinations, which have been spectroscopically characterised. It was therefore of interest to generate mutations which may possibly result in bis Cys or Cys/Met heme ligation, two ligation states which have not been found in nature to date. The A264C and A264M mutants have been successfully generated in both the heme domain and intact flavocytochrome domain and proteins expressed with no noticeable changes to expression.

All proteins appear to be stable, showing no degradation when analysed by SDS-PAGE analysis. Comparison between the Soret maxima of the mutants and the total protein content of pure protein suggest that heme is being fully incorporated at a 1:1 ratio. Therefore the mutations have not impinged on the level of expression of the enzymes or affected their integrity.

Both mutants are capable of binding substrate and inhibitors, although only to around 30 % high-spin in the A264C mutant under saturating substrate concentrations. EPR analysis suggests that there is some Met/Cys heme iron coordination and possibly some Cys/Cys coordination in the A264C mutant. These data has then been contradicted slightly by MCD analysis which suggests that there is slight Met/Cys coordination in the A264M mutant, but no Cys/Cys coordination in the A264C mutant. The discrepancies between the EPR and MCD results can be easily rationalised as an result of the extreme differences in temperature employed for the two techniques, EPR requires temperatures at ~ 10 K while MCD measurement were made at room temperature. Reduction of the A264M mutant has shown the presence of a novel Soret maximum at 435 nm suggesting that on reduction, like the A264H and A264K mutants the thiolate ligand was protonated to give the thiol form. This could possibly arise through interactions between M264 and the heme iron affecting the thiolate bond such that it is destabilised and becomes protonated.
In the flavocytochrome form both mutants were found to be catalytically active, with substrate-dependent NADPH consumption, product formation and low levels of uncoupling, although at rates significantly lower than the wild-type enzyme.

Crystal structures have been obtained for both mutants and show the A264M mutant with M264 coordination, while the A264C mutant does not have C264 coordination. Although the M264 residue is showing some coordination to the heme iron in the crystal structure, EPR and MCD it is also capable of shifting to the high-spin form on substrate-binding to around 70 %, suggesting that the M264 residue is being displaced on substrate-binding. This is interesting since crystallographic analysis does not suggest another conformation which the M264 residue could occupy due to steric hindrance from the other amino acids in the locality, most notably T260 and F87. The A264C mutant structure shows that there is no coordination between the C264 residue and the heme iron, as was seen in MCD analysis. The crystal structure suggested that it is not possibly for C264 coordination to occur without significant structural reorganisation, simply because the cysteine is too short. It is interesting that this mutation is having such a significant effect on the substrate-binding properties and conformation which the enzyme has crystallised without coordinating to the heme iron, possibly due to the changes it is inducing on the amino acids within its locality.

The data presented in this chapter, although not as definitive as that discussed in Chapter 4, allowed spectroscopic analysis which may be of use in the study of novel heme-containing enzymes.
Chapter 6 Characterisation of novel P450 heme ligation states under extremes of pressure
6.1 Introduction

In Chapters 3, 4 and 5 mutations were made at the 264 position of P450 BM3 and the mutants characterised. Each of the mutations showed changes in the properties of the enzyme, with ligations formed between the mutant 264 residue side chain and the heme iron in most of the enzymes analysed. The ligations form to varying extents, and are affected by the heme environment and presence of substrate in certain mutants, but not in others. Certain mutants also exhibit an apparent P450-to-P420 transition on heme iron reduction, as evident from non-standard optical changes. Such changes have been shown to be induced by elevation of hydrostatic pressure in carbon monoxy complexes of P450s (150,265-268). It was of interest to study changes in the heme ligation with increasing pressure, not only in view of the apparent tendency of selected A264 variants to form P420 on reduction, but also due to the possibility that pressure-induced changes in heme iron coordination may be observed for those A264 variants that exhibit novel heme iron ligand sets in the oxidised (ferric) form.

Hydrostatic pressure is a thermodynamic tool that is used to study effects that depend exclusively on volume change. A system which is in at equilibrium between more than one form will shift under pressure to the form with the smaller volume. The volume of the system takes into account three factors, the intrinsic volume of all the atoms, the void volume of the protein due to spaces in the polypeptide packing and the solvation volume which is due to interactions of solvent groups with the polypeptide (269).

Hydrostatic pressure has been used to study a number of P450s, aiding our understanding of the protein's active site. Studies by a number of groups have focussed on changes induced by pressure when the P450 is in the ferrous, carbon monoxide ligated state (150,265-268). It has been shown that increasing pressure will cause the Soret absorbance maximum of reduced/CO ligated P450 to shift from ~ 450 nm to ~ 420 nm, and that reduction of the pressure will return the Soret maximum to ~ 450 nm. The shift from P450 to P420 can also be induced by the addition of salts, denaturants or organic solvents (268,270). However, reversibility of the P450/P420 transition has been achieved by pressure change, but has proven difficult to achieve
using other methods (e.g. by using chaotropic agents). There is, in fact, some uncertainty about the precise chemical nature of the P420 form. It was suggested that the thiol ligand may be replaced by a weaker histidine ligand (150). However, examination of the BM3 crystal structure (1FAG - (135)) shows that there are no histidine residues close enough to replace the proximal thiolate ligand, unless there is substantial structural rearrangement when P420 is formed. It has also been suggested that the P420 form may result from protonation of the proximal thiolate (271), or weakening of the Fe-S bond by lengthening (272). It is now generally considered that the P420 form is a result of protonation of the proximal thiolate ligand to the thiol form. As has been discussed in earlier chapters, the thiolate ligation is essential for catalytic activity in the cytochromes P450, and is required for splitting of dioxygen (34,168).

Davydov et al. have shown that increasing hydrostatic pressure on substrate-bound P450 BM3 causes a shift in the Soret maximum from ~ 390 nm to ~ 418 nm (corresponding to the shift of Soret maxima for a substrate-bound high-spin P450 to that for a substrate-free low-spin P450), followed by a shift to the P420 form of the enzyme (273). Studies by Di Primo et al. have also assessed the effects of pressure on substrate-bound P450 cam. The work by Di Primo et al. has also shown that as pressure is increased the enzyme shifts from the high-spin form to the low-spin form and then to the P420 form. Calculation of the degree of hydration of the active site allowed them to suggest that there is pressure-dependent hydration of the heme pocket which becomes possible by initial pressure-induced displacement of the camphor substrate molecule (267).

The sensitivity observed in the cytochromes P450 to changes in pressure suggested that it may be a valuable method for the study of the A264 mutants of P450 BM3. As high-pressure causes substrate-dissociation in the cytochromes P450, it may also change the equilibrium between the different ligation states in the A264 mutants.
6.2 Results

All A264E pressure studies were carried out at INSERM U-128, CNRS, Montpellier, in collaboration with Dr. Reinhard Lange. All A264K/M/Q/C pressure studies were carried out at the school of Biological and Chemical sciences, Queen Mary University of London in collaboration with Prof. Martin Warren.

High pressure spectra were collected for the heme domains of wild-type and A264E BM3 to examine the pressure effects on the reduced/CO forms and substrate-bound enzymes, allowing comparisons of the wild-type and mutant P450s.

**Figure 6.1** Formation of P420 from P450 in wild type P450 BM3 heme domain

Shown in red is ~ 3 μM CO-bound/reduced wild-type P450 BM3 with its Soret maximum at 449 nm and its α and β-bands at 567 nm and 546 nm, respectively, at 39 MPa. The pressure was increased in approximately 20 MPa increments and the system allowed to equilibrate between each pressure increase. The spectrum collected at the final pressure of 290 MPa is shown in blue and has its Soret maxima at 420 nm and 449 nm.
Figure 6.2  **Effect of pressure on the spectral properties of reduced/CO-bound A264E heme domain.**
The starting spectrum, shown in red, was collected using ~ 6.5 μM A264E heme domain and exhibits a Soret peak at 449 nm, corresponding to a pressure of 3 MPa. The pressure was increased to a final pressure of 290 MPa, at which no further spectral change was observed. At this pressure the Soret band was found at 424 nm. Pressure was increased in ~ 10 MPa increments.

Figure 6.1 and Figure 6.2 show the spectral changes induced, as a function of pressure, for the A264E mutant and wild-type heme domains in the presence of the reductant dithionite and carbon monoxide. By comparison between the two sets of spectra it can be seen that both wild-type heme domain BM3 and the A264E BM3 heme domain behave in essentially the same way. In both cases the starting spectra at near atmospheric pressure have their Soret maximum at 449 nm, and as pressure is applied this P450 Soret maximum decreases, with the appearance of a new maximum at 420 nm, in the case of the wild type enzyme, and at 424 nm with the A264E mutant. The Soret band at 450 nm indicates distally CO-ligated P450 (274). The dominant spectral species in the starting spectrum for A264E is the CO-ligated form and not the glutamate ligated form. As pressure is increased the Soret maximum moves to 420 nm in the wild type enzyme, as has been seen previously for P450 BM3 and for a number of other P450s (266,268,273). The A264E mutant moves to a Soret maximum of 424 nm as pressure is increased. This may suggest that the CO ligand is removed and
replaced by E264 as the pressure is elevated. Soret absorbance in this wavelength range was shown to be indicative of the ferric, glutamate ligated form in Chapter 3. However, given that it is likely that the enzyme remains in the ferrous form throughout the pressure elevation experiment, a more likely scenario may be that the CO ligand remains in place on pressure increase and that protonation of the thiolate occurs, as with wild-type. The shift in absolute position of the P420 Soret from 420 nm (wild-type) to 424 nm (A264E) would then be a consequence of the presence of the charged E264 residue in the immediate vicinity of the CO ligand.

It has been suggested for P450 cam that pressure increase leads to the P420 form of the enzyme as a result of a conformational change that restricts the substrate binding site and/or alters the ligand access channel (150). The structure of the A264E mutant indicated that there are 2 possible conformations for the glutamate, the unligated form, where the glutamate residue sits at the base of the substrate access channel, against F87, and the second form where it ligates to the heme iron. If the substrate access channel is compressed then this will probably force the glutamate to ligate to the iron, causing the CO to be displaced. Both the wild type and A264E mutant show near complete conversion back to the P450 form when pressure was returned to near the starting pressure.

A further point to note regarding the comparative behaviour of the wild-type and A264E CO complexes is the fact that near-complete conversion to the P420 form occurs in the case of the A264E variant at 290 MPa, whereas there is apparently only an ~60 % conversion to P420 for the wild-type heme domain.
Chapter 6  Effect of pressure on wild type and mutant P450 BM3

Figure 6.3  Pressure effects on wild-type heme domain of P450 BM3 in the presence of saturating arachidonate.
The starting spectrum, collected at 2.7 MPa (in red), is a typical arachidonate-bound BM3 spectrum (~2.5 μM) with its Soret peak at ~393 nm. Subsequent spectra were collected at pressure increments of 20 MPa, with full equilibration at each point. The final point was reached at a pressure of 220 MPa, has the Soret peak at 418 nm and is shown in blue.
The effect of pressure on the spectral properties of arachidonate-bound A264E heme domain

Spectra were recorded for 3.5 μM A264E heme domain containing 10 μM arachidonic acid with 5 minutes equilibration before collecting each spectrum. The starting spectrum, shown in red, was collected at 5 MPa. The Soret band maximum is at 426 nm and the α and β-bands are at 575 nm and 542 nm, respectively. The final spectrum collected after no further absorbance changes were observed is shown in blue and corresponds to a pressure of 325 MPa, with the Soret maximum at 419 nm and a shift in the β-band to 536 nm. Spectra between were collected at intervals of approximately 40 MPa. A number of intermediate spectra are shown in black. The spectrum shown in green was collected after the pressure was returned to ~ 5 MPa, and shows a Soret maximum at 414 nm, with α- and β-bands at 536 nm and 534 nm respectively.

Figure 6.3 and Figure 6.4 show the spectral changes caused by increasing pressure on the heme domains of substrate-bound wild type BM3 and the A264E mutant. Typically, the addition of substrate to a cytochrome P450 will cause the Soret maximum to shift from ~ 418 nm to ~ 390 nm. This shift in Soret maximum correlates to the displacement of the distal water molecule by the substrate and a corresponding shift in the heme iron from the low- to high-spin form. As was shown in Chapter 3 in the A264E mutant, binding of substrate causes the E264 residue to become almost fully ligated to the heme iron, and crystallographic studies have shown that the enzyme
is in the substrate-bound conformation even in the absence of substrate. In studies of spin-state transitions under pressure, Davydov et al. have shown that increasing pressure leads to a positive volume change in the hydration pocket of palmitate-bound P450 BM3, causing enzyme to move to the low-spin form, but maintaining the palmitate in the active site. They suggest that the reason for this is increased hydration of the active site, possibly with the water forming the proximal ligand, although there is a palmitate molecule present (273). Di Primo et al. have also studied the effects of pressure on heme-pocket-hydration in P450 cam and suggest that the differences seen in the activation volume between substrate-bound and substrate-free P450 cam can be attributed to changes in the hydration of the active site. In the substrate-bound form the substrate is apparently displaced by water as the pressure is increased (267). This can explain the spectral shifts seen as a function of pressure increase with the A264E mutant and wild-type BM3, both having the distal water ligand at high pressure when the active site volume is decreased. The wild-type enzyme shows conversion back to the high-spin form following reduction of the pressure, suggesting that the water molecule is again displaced from the heme iron in the presence of substrate and the enzyme returns to the high-spin form. The decreased active site volume, and introduction of water molecules into the active site as pressure is increased, likely result in displacement of the E264 ligand to the heme iron. The spectral features suggest that water may replace the E264 side chain as a ligand to the heme iron at high pressure. The fate of the fatty acid substrate is not clear at this point. It may either be excluded from the active site, or remain bound but in a binding mode which still enables water to ligate at the distal position. On reduction of the pressure back to atmospheric pressure the A264E mutant does not return to the form seen prior to the application of pressure. Instead, the Soret maximum shifts to 414 nm, appearing to be a single species without any significant shoulders that might indicate equilibrium between different forms. The position of the Soret suggests a low-spin form of the heme iron, and may suggest that the heme iron is entirely ligated by water rather than by the E264 side chain. The structural origin of this final species remains undefined, but is clearly a phenomenon that should be investigated further by spectroscopic methods. Possibly, given the known conformational plasticity of the enzyme, a structural reorganisation of the P450 distinct from that seen for the wild-type enzyme occurs on pressure decrease with the A264E heme domain.
In a further attempt to characterise the new spectral signal seen for the A264E mutant following exposure to high pressure, spectra were also collected for the enzyme in the absence of substrate and in the presence of the reductant dithionite.

![Spectroscopic effects of pressure on the reduced A264E mutant P450 BM3 heme domain](image)

**Figure 6.5** Spectroscopic effects of pressure on the reduced A264E mutant P450 BM3 heme domain

Pressure was increased from 40 MPa to 297 MPa in ~ 20 MPa intervals, allowing equilibration between collection of each spectra. The blue spectrum is the starting spectrum of ~ 5 μM reduced A264E heme domain at 40 MPa, with a typical reduced spectrum (Soret peak at 412 nm and fused α- and β-bands). The red spectrum was collected at 297 MPa and shows the Soret peak at 427 nm, with the α-peak at 560 nm and the β-peak at 529 nm. Isosbestic points can also be seen at 418 nm, 444 nm, 521 nm, 535 nm, 525 nm and 568 nm.

The spectral changes associated with increasing pressure for the dithionite-reduced A264E mutant are shown in Figure 6.5. The starting spectrum is typical of a ferrous P450, with its Soret maximum at 412 nm and fused α- and β-bands. The pressure-induced shift in the Soret maximum to 427 nm and α- and β-bands from a single fused species at 560 nm to discrete bands at 560 nm and 529 nm, respectively, is indicative of protonation of the proximal thiolate cysteine ligation resulting in thiol ligation to ferrous heme, as was demonstrated by Perera et al. (164) and discussed in Chapter 4.
The apparently complete spectral conversion to the thiol-ligated form of A264E provides a useful tool for "clean" conversion to this species, and should allow for further spectroscopic characterization of this form, including determination of extinction coefficient of \(~ 42 \text{ mM}^{-1} \text{ cm}^{-1}\) at 412 nm. Constraints of time have not allowed collection the equivalent analysis to be made of the wild-type enzyme, but it in light of pressure effects on CO bound ferrous wild type P450 BM3 it follows that in the ferrous form without CO present the enzyme would shift to the protonated thiol form as pressure is increased.

The spectral changes seen in the wild-type enzyme in the presence of CO or substrate as the pressure of the system is increased show that the axial ligands to the heme iron can be modified by pressure. With this in mind each of the mutants was studied further, using increasing pressure as a possible way to induce changes to the heme ligation state.
Figure 6.6  Effect of pressure on oxidised wild-type P450 BM3

Shown in red is the starting spectrum of typical low-spin oxidised wild-type P450 BM3 heme domain with its Soret maximum at 414 nm, α-band 568 nm at and β-band at 535 nm. The pressure was increased in 50 MPa increments and the system allowed to equilibrate at each stage. Two intermediate spectra are shown in black. The final spectrum collected, shown in blue, was collected at a pressure of 646 MPa and has its Soret maximum at 426 nm with the α-band losing intensity and the β-band shifted to 545 nm.

Davydov et al. (273) also investigated the effects of pressure on the spectral properties of substrate-free wild-type P450 BM3, observing a decrease in the intensity of the Soret signal with increasing pressure and a spectral shift virtually the same as that shown above in Figure 6.6. They attributed the form seen at high pressure to the same species observed in the ferrous-CO complex – i.e. the P420 form. This, in turn, is due to the switch from thiolate to thiol ligation from the proximal cysteine. In this case, the switch occurs in the ferric form of the enzyme rather than the ferrous. Comparison between the data shown in Figure 6.6 and Figure 6.4 shows that the wild-type enzyme under high-pressure and A264E mutant in the presence of substrate following the reduction of the pressure to ambient have the same spectral form with Soret maximum at 414 nm. This pressure has resulted in nonreversible formation of the protonated
proximal cysteine residue, possibly by increasing the pressure past a critical point after which reversibility is no longer possible in the A264E mutant.

Figure 6.7 **Effect of pressure on the A264E heme domain of P450 BM3**

Spectra were recorded for 5.5 μM A264E heme domain in the absence of any substrate or ligand. The starting spectrum, shown in red, was collected at 12 MPa and has a Soret peak at 421 nm, with its $\alpha$-band and $\beta$-band at 539 nm and 570 nm, respectively. The final spectrum, collected at 260 MPa, is shown in blue and has its Soret maximum at 424 nm and a maximum in the visible region at 541 nm. Spectra were collected at ~50 MPa intervals, with a number of intermediate spectra shown in black.

The initial spectrum shown in Figure 6.7 has its Soret maximum at 421 nm, and shifted around 2 nm at the first pressure increase (48 MPa). This shift has been observed previously and attributed to small changes in position of the distal water molecule (275). The same slight shift was observed in all mutants and wild-type pressure studies where water is the initial distal ligand.
Figure 6.8  **Effect of pressure on the A264H P450 BM3 heme domain**

Spectra were collected for ~ 6.5 μM A264H heme domain, with the initial spectrum collected at 16 MPa (shown in red) having its Soret maximum at 427 nm, α-band at 543 nm and β-band at 578 nm. The final spectrum is shown in blue, corresponds to a pressure of 645 MPa, and has its Soret maximum at 426 nm. The pressure was increased in ~ 50 MPa increments, a number of intermediate spectra have been removed for clarity.
Figure 6.9  Effect of pressure on the A264K P450 BM3 heme domain  
Shown in red is the starting spectrum for ~6.5 μM A264K heme domain. The spectrum was collected at 19 MPa and has its Soret maximum at 425 nm, its α-band at 574 nm and β-band at 543 nm. The pressure was increased in ~50 MPa increments the final spectrum, collected at 648 MPa, is shown in blue and has its Soret maximum at 425 nm, with its α-band diminished and β-band at 543 nm. Intermediate spectra have been removed for clarity.
Figure 6.10  **Effect of pressure on the A264Q P450 BM3 heme domain**

Shown in red is the starting spectrum collected for ~6.5 μM A264Q heme domain at a pressure of 17 MPa, with its Soret maximum at 419 nm, α-band at 534 nm and β-band at 567 nm. The final spectrum, collected at 595 MPa, is shown in blue and has its Soret maximum at 424 nm and its β-band at 542 nm. Spectra were collected at 50 MPa intervals and two examples are shown in black.

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Chapter 6  **Effect of pressure on wild type and mutant P450 BM3**
Figure 6.11  Effect of pressure on the A264M P450 BM3 heme domain
The spectrum, shown in red, is the starting spectrum collected at 20 MPa for 6 μM A264M heme domain with its Soret maximum at 418 nm, α-band at 568 nm and β-band at 536 nm. The final spectrum, shown in blue, was collected at 595 MPa, with its Soret maximum at 426 nm, diminished α-band and β-band at 545 nm. Spectra were collected at 50 MPa increments and showed a clean conversion between the two forms. For clarity intermediate spectra are not shown.
The effects of pressure on the substrate-bound wild type enzyme is shown in Figure 6.3. Comparison between the wild-type and A264M mutant shows that the A264M mutant behaves in essentially the same way as the wild-type, shifting from the high- to low-spin form as pressure is increased. The resultant Soret maximum at 424 nm in the A264M mutant contrasts with the Soret maximum seen in the wild type mutant which is at the typical wavelength for substrate-free wild type P450 BM3 (418 nm). It is possibly indicative of presence of the distal water ligand but with the M264 residue affecting it such that the Soret maximum is shifted to 424 nm. As was discussed above Davydov et al. have analysed the effects of pressure on substrate-bound P450 BM3 and suggested that the spectral shift observed is due to increased hydration of the active site, while maintaining the substrate in the active site (273). There is a charge transfer band at ~ 650 nm in the A264M mutant which is indicative of substrate bound, this also becomes diminished as pressure is increased, again suggesting that it becomes
replaced by water. There also appear to be 2 isosbestic points, initially at 400 nm and then at 409 nm, this suggests that there are 2 events occurring as pressure is increased, most probably firstly the displacement of the substrate molecule and secondly the protonation of the proximal cysteine residue to the thiol form as is seen in substrate-free A264M in Figure 6.11.

![Graph showing the effect of pressure on the A264C P450 BM3 heme domain.](image)

**Figure 6.13** **Effect of pressure on the A264C P450 BM3 heme domain**

Shown in red is the starting spectrum for ~4.5 μM A264C at 14 MPa, with Soret maximum at 419 nm, α-band at 567 nm and β-band at 534 nm. The final spectrum collected is shown in blue and corresponds to a pressure of 597 MPa Its Soret maximum is at 424 nm and the β-band is at 544 nm. The pressure was increased in ~50 MPa increments; a number of the intermediate spectra are shown in black.

Comparison between the effects of pressure on each of the A264 mutants in the substrate-free ferric form has shown that they all behave in essentially the same way. As pressure is increased the Soret maximum shifts ultimately to ~424 nm with the α-band losing intensity and the β-band shifting to around 545 nm. This is attributed to formation of ferric thiol-ligated heme iron, *i.e.* the protonation of the cysteinate ligand and the ultimate formation of P420. Following the increasing pressure studies pressure was returned to ambient pressures in each experiment. With the exception of substrate-bound A264 each time the Soret maximum shifted back to the starting
Chapter 6  Effect of pressure on wild type and mutant P450 BM3

spectrum in almost all of the sample. In each case there was a small proportion which did not change on reduction of pressure, suggesting that it had become inactivated, possibly with the pressure having induced structural changes which prevented the enzyme from returning to the initial form. The A264K/H/Q/C mutants were not analysed in the presence of substrate as time constraints did not allow analysis of all the mutants in the presence of substrate and these mutants showed much less or no spectral changes on addition of substrate.
6.3 Discussion

This section of work has studied the effects of pressure on wild-type P450 BM3 heme domain and on the A264E/K/H/Q/M/C mutant heme domains in various forms and redox states (according to availability of protein and access to instruments, as time allowed). Wild-type and A264E BM3 show reversible formation of the P420 form from the reduced/CO bound P450 form. In the presence of substrate (arachidonic acid) the wild-type, A264E and A264M forms undergo shifts from the substrate-bound form (which is predominantly high-spin, aqua-ligated in wild-type and A264M enzymes, and is E264 ligated form in A264E) to spectral forms indicative of the low-spin substrate-free form. These results concur with those previously obtained for P450 BM3 by Davydov *et al.* (273) and P450 cam by Di Primo *et al.* (267) in that removal of substrate from the P450 active site is considered to be a consequence of pressure elevation with the substrate-bound forms. In the reduced form without CO present, the proximal thiolate has become protonated to the thiol form in the A264E mutant as pressure is increased, producing large optical changes in each of the three major heme bands (α, β and Soret). These data concur with the commonly held opinion that the P420 form of the cytochromes P450 is a result of protonation of the thiolate proximal ligand to the thiol form when the enzyme is in the reduced ferrous state with CO as distal ligand. Thus, pressure studies reported here provide spectral characterisation of the P420 form of the P450 BM3 enzyme in the absence of the CO ligand.

The spectral shifts associated with increasing pressure in wild-type and each of the A264 mutants in their oxidised forms concur with protonation of the cysteine proximal to the heme iron, leading to a switch from thiolate to thiol coordination of the iron. As pressure is increased the active site volume is decreased and water molecules are likely to be forced into the P450 BM3 active site (267,273).

In the substrate-bound forms studied here (*i.e.* wild-type, A264E and A264M) optical changes indicative of the displacement of substrate (arachidonate) are observed as pressure is increased. In the case of the wild-type enzyme data concur with those obtained by others (267,273) and have been attributed to a high- to low-spin shift as pressure is increased, due to increased hydration of the active site resulting in water forming distal ligand although it is thought substrate is not expelled from the active
Chapter 6 Effect of pressure on wild type and mutant P450 BM3

site. The A264M mutant showed similar shifts, moving from a high-spin form to a new form with Soret maximum at 424 nm, which is probably a result of water as distal ligand with the M264 residue affecting the water. For the substrate-bound form of the A264E heme domain, the spectra occurring on pressure increase are indicative of the displacement of the E264 ligand to the heme iron, which in turn is likely to reflect (at least in part) displacement of the arachidonate from the active site environment. However, the final position of the Soret band is at 419 nm, close to that for the wild-type P450 BM3, but distinct from that of the A264M enzyme. Moreover, the position of the Soret band at high pressure is very different for the A264E enzyme according to whether substrate is bound (419 nm) or not (424 nm). Clearly there are important pressure induced differences observed in the optical properties of the various wild-type and mutant enzymes, and the binding of substrate can influence the nature of the final species formed (perhaps either by remaining bound at a position separated from the active site or by influencing the conformation of the starting species and perhaps the pathway of subsequent conformational change on pressure elevation).

These studies of pressure induced spectral changes in the various forms of wild-type and A264E P450 BM3 heme domains were limited by time and access to the relevant instruments. However, they have been informative with respect to observing a number of interesting features of the P450 system. Pressure elevation clearly provides an elegant route to forming the P420 species (i.e. the thiol-ligated form of the heme iron), both in ferric and ferrous forms of the enzyme. In the case of the ferrous (non CO-ligated form) it produces a clean spectrum with sharp $\alpha$, $\beta$ and Soret features that characterise this species. In general, pressure-induced spectral transitions are rather "clean" (usually with distinct isosbestic points), although in the presence of substrate there is at least some evidence for substrate dissociation (or movement away from the heme environment) at pressures lower than those required for thiolate protonation with P420 formation (i.e. spectral transitions observed between more than two species). Differing spectral characteristics of the "end point" species collected at high pressure are also indicative of structural and conformational differences between the various enzymes, and may also indicate that different pathways of pressure-induced protein deformation occur (although a precise structural description of these events is beyond the scope of this work).
In conclusion, pressure increases for P450 BM3 and its mutants in various forms provide novel routes to production of the thiol-ligated P420 species and for inducing displacement of substrate from the active site around the enzyme. This technique thus has potential for generating novel P450 and P420 species for more detailed spectroscopic characterization. Within the time frame available for this work, it was not possible to establish whether the method could be useful in inducing changes in the distal ligands to the heme iron (beyond the likely formation of a low-spin aqua-coordinated species from 5-coordinate high-spin, substrate-bound forms as pressure is increased). However, further studies (perhaps within a narrow range at pressures only moderately above ambient atmospheric pressure) may provide a route to altering distal ligand occupancy in e.g. the substrate-free A264E enzyme or the A264M/Q variants. High pressure thus has the potential to be a valuable technique in the production of novel states of P450s and mutant forms thereof, and possibly in the production of different conformational states of P450s – including those states which this work has shown through A264E engineering of P450 BM3 can be differentially populated according to either substrate ligation or mutagenesis of the I helix.
Chapter 7  Conclusions and future perspectives
In this thesis I have discussed generation and characterisation of (by structural, kinetic, spectroscopic and thermodynamic methods) a number of mutants of flavocytochrome P450 BM3 and its heme domain. Initially the A264E mutation was made to P450 BM3 in both the heme domain and intact flavocytochrome forms of the enzyme with the aim of generating a heme covalently ligated to the protein backbone via a linkage between the glutamate carboxylate and the heme 5-methyl group. This aim was not fulfilled, a major reason for which appears (from structural analysis of the A264E heme domain) to be steric obstruction by the side chains of amino acids F87 and T260. However, a novel heme ligation state was instead observed for the A264E enzyme and fully spectroscopically and structurally characterised (225).

The structure of the A264E mutant in both substrate-free and palmitoleate-bound forms showed the enzyme in the substrate-bound conformation previously thought to be restricted to fatty acid-bound forms of the P450. This challenged the previously held theory that the displacement of the water held as a distal ligand to the heme iron was responsible for a conformational change in the enzyme. In turn, these data suggested that the enzyme is actually in an equilibrium between a number of forms in solution, and for the wild-type enzyme in the absence of substrate, the predominant conformer is that previously described as the "substrate-free" (SF) state. For the wild-type, the conformational equilibrium shifts towards the substrate-bound form (SB) on binding of substrate, and dogma in the field suggested that the binding of the substrate induces the structural change. The fact that A264E adopts the SB conformation in both fatty acid-free (in which there is partial coordination of the heme iron by E264) and fatty acid-bound forms (in which there is complete coordination) suggested that the fatty acid binding per se might not be the key to the SF-to-SB conformational transition. Instead, a likely scenario is that the P450 occupies two (and possibly more) predominant conformers in solution, with differing affinities for substrate. The SB conformation has higher substrate affinity than does the SF conformation. The I-helix mutation A264E leads to an alteration in the equilibrium (regardless of the presence or absence of substrate) in favour of the SB conformation. Fatty acid binding titrations then demonstrated that the A264E heme domain has significantly higher affinity for fatty acid than does the wild-type P450. Thus, studies of the A264E enzyme produced not only a novel, substrate-induced P450 heme iron ligand set, but also gave rise to a more complete understanding of conformational transitions in the P450, how they can
be structurally triggered and the influence of conformational changes on substrate affinity (148).

The A264K/H/Q/M/C mutants have also been successfully generated in the heme domain and flavocytochrome constructs, and characterised by the same battery of spectroscopic, kinetic and structural methods as employed for the A264E variant. The A264K and A264H mutants showed complete Cys/Lys and Cys/His heme iron ligation. Both A264K and A264H are catalytically inactive, with no fatty acid substrate binding detectable by optical titration, or hydroxylated fatty acid product formation. In the case of substrate binding titrations, it appears obvious that fatty acids can still bind to the A264H/K P450s. However, since they are unable to displace the coordinating amino acid side chain ligands from the heme iron, their binding is spectrally silent. Spectroscopic analysis of the A264Q/M/C mutants has shown Cys/Glu, Cys/Met and probably Cys/Cys ligation to some extent in each mutant. All of these mutants retained catalytic activity, albeit at lower rates than the wild-type P450 BM3. These mutants have allowed characterisation of further novel forms of axial heme ligation, and data collected using (in particular) EPR and MCD spectroscopy has contributed further to the generation of a spectroscopic “ruler” by which different types of heme ligations can be classified and which may be of use for the recognition and elucidation of novel and pre-existing types of axial heme ligation in newly isolated cytochromes. Structural analysis of these mutants was in broad agreement with data collected from spectroscopic analysis, and showed that all mutants with the exception of A264C had axial ligation between the new 264 residue side chain and the heme iron. In the case of the A264C mutant, spectroscopic analysis indicated that partial coordination of the heme iron is likely to occur in solution, but structural analysis indicated that any such coordination must involve a conformational state distinct from those characterised to date. In turn, this may suggest that P450 BM3 heme domain can occupy further, as yet structurally undefined, conformation(s) in which steric obstructions are relieved and in which distance constraints relaxed in order to permit bis-cysteinate heme iron ligation for the A264C enzyme. All except one of the mutant structures are solved in the SB conformation without the addition of exogenous fatty acid substrate. However, the A264H mutant is in the SF conformation. The reasons for the distinct conformation in the case of A264H (relative to other A264 variants) deserves further exploration. In addition to the spectroscopic
"ruler" generated through analysis of the A264 mutants using EPR and MCD methods, the A264H and A264K mutants have generated novel, hexacoordinated forms of the enzyme which have virtually no catalytic activity with respect to fatty acid hydroxylation. This almost certainly reflects the near-complete coordination of the heme iron by Lys and His side chains in both cases, and the inability of the fatty acids to displace these ligands in favour of molecular oxygen to enable oxygenation chemistry to occur. The flavocytochrome forms retain full electron transferase activity within their reductase domains – as evidenced by high levels of ferricyanide and cytochrome c reductase activity. This has been exploited by Neeli et al., who have used the inactive A264H flavocytochrome P450 BM3 together with the flavin-depleted G570D mutant, which does not contain a FMN cofactor, to show that flavocytochrome P450 BM3 is functional as a fatty acid hydroxylase as a dimer (110). Thus, productive electron transfer within a monomer cannot be achieved from NADPH to heme iron in G570D, due to the absence of the FMN. It cannot occur in A264H due to the hexacoordinated heme iron. However, it can occur in the dimer as a consequence of electron transfer from the FMN of the A264H mutant to the heme of the G570D mutant. Thus, electron transfer occurs between monomers in a P450 dimer to facilitate fatty acid hydroxylation in P450 BM3. The same type of phenomenon occurs in P450 BM3's flavocytochrome relative, nitric oxide synthase (276). In future work, it may also be of interest to utilise the A264K and A264H mutant forms (which are both inactive but populate different conformations) to assess the effect heme domain conformation has on inter-flavin electron transport. In previous studies Murataliev and Feyereisen demonstrated that fatty acid binding to the wild-type flavocytochrome enhanced the rate of electron transfer from the reductase domain to cytochrome c (277). The A264H and A264K mutants may provide the basis for examining this phenomenon in terms of the influence of structural conformation of the heme domain (which differs between SB and SF for these two mutants in absence of any bound fatty acid) on inter-cofactor electron transfer.

Over recent years interest has arisen in the biotechnological exploitation of enzymes to catalyse reactions for which current chemical methods are unsatisfactory. Often, chemical production gives poor yield, non-specific racemic mixtures, production of harmful by-products or uses undesirable reaction conditions (for example extremes of heat or pressure). In such scenarios, enzymes have the benefit of (often) near-complete
Conclusions and Future Perspectives

Conversion of substrates to one or a small number of products, and they are generally used at ambient temperatures and pressures. A drawback, of course, is the relative instability of many enzymes in continuous use. This was a major factor considered at the outset of this work, since it was suggested that cross-linking the heme to the protein backbone might result in enhanced stability of its binding and in less formation of P420, while maintaining wild-type-like catalytic properties. In fact, a number of groups have tried to optimise P450 BM3 for biotechnological use (171, 205, 278, 279). These groups have attempted to change substrate specificity from long chain fatty acids towards short chain alkanes, indole rings and phenolics using both site-directed mutagenesis and forced evolution methods. It has also been shown previously that P450 BM3 is capable of epoxidation reactions (180) and this is an area which has been little explored to date, particularly with regards to optimisation towards bioactivation by epoxidation of compounds such as styrene. In addition to optimising substrate specificity, there are a number of problems which hinder P450 BM3's biotechnological optimization. Two major factors are: firstly the requirement for the costly hydride donor NADPH, and secondly the instability of the heme cofactor itself (particularly with respect to its conversion to the inactive P420 form). Changes to P450 BM3's obligate requirement for NADPH as an electron donor have been achieved by a number of groups. Using directed evolution on the heme domain of P450 BM3, Cirino et al. optimized P450 BM3 towards peroxide-driven catalysis by the peroxide shunt mechanism. However, these authors observed significantly reduced rates of reaction using peroxide to drive the enzyme turnover when compared with the wild-type enzyme with NADPH as hydride donor. They also found stability problems with the enzyme when it was exposed to a high concentration of hydrogen peroxide (208). Unfortunately, high levels of peroxide are required for a more effective peroxide shunt reaction — but such conditions also result in the oxidative destruction of the heme macrocycle and in the covalent modification of the protein itself. While the peroxide shunt is an interesting means of driving P450 reactions, it is doubtful that it can be used effectively for an enzyme such as P450 BM3 without extensive engineering to improve dramatically the stability of the enzyme towards peroxide and to enhance the efficiency by which the peroxide interacts with the heme to effect better catalysis. Neeli et al. successfully switched the specificity of the hydride donor from NADPH to the cheaper NADH by mutating the FAD domain residue W1046 to alanine or histidine (280). The W1046 residue "shields" the FAD isoalloxazine ring and is a key

278
Conclusions and Future Perspectives

determinant of pyridine nucleotide specificity. Removing this shield dramatically improves efficiency of hydride transfer and binding with NADH. The final major hurdle to the utilisation of P450 BM3 for biotechnological purposes is the instability of the heme. In this work an initial aim was to facilitate a stabilising heme-protein link by engineering the A264E mutation. However, the structure of the A264E mutant heme domain showed that the apparent failure of the mutant to form covalent ligation to the heme macrocycle may be due to steric hindrance from T260 and F87 – preventing access of the E264 carboxylate to the relevant heme methyl group. Losing this steric bulk by creating the T260S and/or F87G mutations in conjunction with the A264E mutation may allow the E264 residue to reach the 5-methyl group of the heme and form the covalent ligation during enzyme turnover. Additionally, generation of the F261E mutation may also be an alternative route to generation of the covalent ligation. F261 resides one turn further up the I helix than does A264, and a glutamate engineered at this position may be able to access the 5-methyl group without steric hindrance by T260 or F87. In addition, if the residues F86 and I401 were mutated to glutamate they may come close enough to the methyl groups of the heme that ligation under catalytic conditions could occur. Thus, further routes to the generation of covalent linkages between protein and heme macrocycle exist – and should be the subject of further research with the long term aim of generating a more robust, biotechnologically-exploitable oxygenase catalyst from P450 BM3.

This thesis has shown the generation and characterisation of a set of mutants in flavocytochrome P450 BM3, a model system in the P450 superfamily of enzymes and a model enzyme for rationalizing P450 function and electron transfer. Various mutations at position 264 produced variants with completely novel heme iron ligation states, providing important spectroscopic “benchmarks” that will be of importance in futures studies of heme-containing enzymes. Moreover, kinetic, spectroscopic and structural analyses of the A264E variant have produced important data challenging the previously held conceptions of conformational equilibria in the P450, their origin and relevance to catalysis. Studies of the effects of high pressure on wild-type and A264 mutant P450s have also demonstrated that these enzymes can be cleanly converted to P420 species in the oxidised state – providing clear spectral signatures for these species. This provides an elegant method for studying the biochemical of the P420 form (which results from protonation of the cysteine thiolate).
Collectively, these studies have provided novel data on a key enzyme in the P450 superfamily relating to production of novel ligation states and an advanced understanding of conformational equilibria. They provide the platform for more detailed studies to further deconvolute structure/mechanism relationships in P450 BM3 (and, more generally, in the P450 family as a whole).
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282


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305


The flavocytochrome P450 BM3 Mutant A264E Undergoes Substrate-dependent Formation of a Novel Heme Iron Ligand Set*

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A conserved glutamate covalently attaches the heme to the protein backbone of eukaryotic CYP450 enzymes. In the related Bacillus megaterium P450 BM3, the corresponding residue is Ala264. The A264E mutant was generated and characterized by kinetic and spectroscopic methods. A264E has an altered absorption spectrum compared with the wild-type enzyme (Soret maximum at −420.5 nm). Fatty acid substrates produced an inhibitor-like spectral change, with the Soret band shifting to 426 nm. Optical titrations with long-chain fatty acids indicated higher affinity for A264E over the wild-type enzyme. The heme iron midpoint reduction potential in substrate-free A264E is more positive than that in wild-type P450 BM3 and was not changed upon substrate binding. EPR, resonance Raman, and magnetic CD spectroscopies indicated that A264E remains in the low-spin state upon substrate binding, unlike wild-type P450 BM3. EPR spectroscopy showed two major species in substrate-free A264E. The first has normal Cys-aqua iron ligation. The second resembles formate-ligated P450cam. Saturation with fatty acid increased the 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-infrared magnetic CD 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 (Joyce, M. G., Girvan, H. M., Munro, A. W., and Leys, D. (2004) J. Biol. Chem. 279, 23287–23293).

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Novel Heme Ligation in a Cytochrome P450

CYP 102 (B. megaterium) 253 IRYQ11TFLIAGHETTSGLLSFALYFLVE
CYP 4A4 (Rabbit) 310 LRAEVDTMGPDFGTASVSWIFYALAT
CYP 4A1 (Rat) 309 LRAEVDTMGPFHDTASVSWIFYALAT
CYP 4B1 (Human) 304 LRAEVDTMGPFHDTTTSGISWFLYCMAL
CYP 4C1 (Cockroach) 303 IREEVDTMGPFHDTTSAICWALLGSS
CYP 4D1 (Drosophila) 305 IREEVDTMGPFHDTTSSALMFFFFFYNIAT
CYP 101 (P. putida) 238 AKRMCGLLLVGCGLDVNFLPSMFLAK

Fig. 1. Alignment of P450 amino acid sequences in the region of the heme-ligating glutamate in the CYP4 family. An alignment of amino acid sequences from various members of the CYP4 family of fatty acid-oxygenating P450 enzymes was performed using the ClustalW sequence alignment program via the European Bioinformatics Institute (available at www.ebi.ac.uk/clustalw/). The sequence alignment shown is for residues in the I helix region of the P450 enzymes, 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 shown in underlined. The amino acid sequences shown are for flavocytochrome P450 BM3 from B. megaterium (CYP4A1), rabbit CYP4A4, rat CYP4A1, human CYP4B1, cockroach CYP4C1, Drosophila melanogaster CYP4D1, and Pseudomonas putida P450cam (CYP101).

thermal, or pressure treatment (see Refs. 16 and 17) to generate one or more inactive forms with altered electronic spectral properties, the so-called "P420" species. In the P420 enzyme, 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 ~420 nm (in the inactive form). Protonation of the thiolate ligated is thought to underlie this spectral change and concomitant loss of activity (18). Heme can also be dissociated from P450 enzymes under harsh conditions, and its re-incorporation may not be facile (19). Given the attention to the biotechnological exploitation of P450 enzymes in, for example, diagnostics, bioensing, and fine chemical synthesis (see Ref. 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 their operational stability remains a major problem.

Recent studies on mammalian family 4 (fatty-acid hydroxylase) P450 enzymes have revealed unusual stability in the binding of heme in various members of the family. It was shown that SDS-PAGE resolution of 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 (Glu318 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 enzyme 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 is autocatalytic and occurs because of esterification with the glutamate via the heme 5-methyl group (25). The formation of a porphyrin carboxylation 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 recognized in any other P450 isoform, but the ability to stabilize the heme cofactor may have important ramifications regarding the exploitation of P450 enzymes in such areas as biotransformations and toxicological applications.

Flavocytochrome P450 BM3 has been recognized 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 catalyze regio- and stereoselective hydroxylation and epoxidation 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 (see Refs. 21, 22, 27, and 28). However, P450 BM3 exhibits the same structural instability observed in other P450 isoforms regarding 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 suggests that covalent heme linkage might also be feasible in this isofor, and the fact that it has not been observed for wild-type P450 BM3 could be explained by the presence of an alanine rather than a glutamate in the respective position of the conserved I helix region in this P450 enzyme (Fig. 1) (29).

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 sixth 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 enzyme.

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 proteins and heme domains (aminos acids 1-472) were performed essentially as described previously (30, 31). Expression plasmids pHM20 and pHM22 (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 in Escherichia coli strain TG1 grown in Terrific Broth and 50 µg/ml ampicillin (typically 5 liters of cells) for ~36 h following inoculation from an overnight culture of the relevant transformant. Cells were collected by centrifugation, resuspended in buffer A (50 mM Tris-HCl (pH 7.2) and 1 mM EDTA), and broken using a French press (three passes at 950 psi), followed by sonication of the resulting suspension on ice in a Bandelin Sonopuls sonicator (5 × 20-s 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 (both 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-colored fractions were retained, concentrated by ultrafiltration (Centriprep 30, Millipore Corp.), and dialyzed extensively into buffer B (25 mM potassium phosphate (pH 6.5)) containing...
benzamidine and phenylmethylsulfonyl fluoride. Intact flavocytochrome P450 BM3 and its A264E mutant were loaded onto a mimitic yellow column (2 × 15 cm) and washed extensively with buffer B prior to elution with 25 mM 2'- and 3'-AMP (mixed isomers, Sigma) containing 500 mM potassium phosphate (pH 6.5) plus protease inhibitors. The most intensely red-colored fractions were pooled, concentrated, and exchanged by dialysis into buffer A at 4 °C prior to loading onto 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 use in buffer A and 50 mM (v/v) glycerol and storage at −80 °C. The A264E heme domain used in crystallographic trials was exchanged instead into 10 mM Tris-HCl (pH 7.2) and used directly for crystalllogenesis.

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 chromophore fragment and pBM20 template in PCRs A and B. A 518-bp fragment construct contains the −1.4-kb gene encoding the heme domain of P450 BM3 (amino acids 1–472 of the flavocytochrome) in the expression vector pUC18 (30). PCR A used primers MfF (5'-CGGTTTGGCGGTTGGCCG-3', incorporating an MfeI restriction site underlined) and BamR (5'-CCTAGCTGAAATCCGGG-3', encompassing the mutagenic codon, with the mutated nucleotide underlined). PCR B used the same BamH1 and primers BMfF (5'-CTTCAATTGGTCTTTGCGG-3', incorporating a BamHI restriction site underlined 335 bp downstream of the mutation). The final PCR (PCR C) combined the products of PCRs A and B using primers MfE and BamR. The product of PCR C was A-tailed using Tag DNA polymerase and ligated into pGEM-T (Promega pGEM-T Easy Vector Systems) according to the manufacturer's instructions. This plasmid was digested with the same restriction enzymes and gel-purified to yield a 518-bp fragment containing the A264E mutated region. The relevant fragment was added to an agarose gel and purified using a QIAquick gel extraction kit (QIAGEN Inc.). The MfeI/Msel restriction fragment was ligated into the backbones of plasmid pMG20 and pBM23, which 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 site 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 the purity of the wild-type and mutant flavocytochrome P450 BM3 enzymes and heme domain and also to resolve the A264E flavocytochrome P450 BM3 enzyme and heme domain prior to testing for covalent attachment between the heme and flavocytochrome P450 BM3 enzymes. The final PCR (PCR C) combined the products of PCRs A and B using primers MfF and BamR. The product of PCR C was A-tailed using Tag DNA polymerase and ligated into pGEM-T (Promega pGEM-T Easy Vector Systems) according to the manufacturer's instructions. This plasmid was digested with the same restriction enzymes and gel-purified to yield a 518-bp fragment containing the A264E mutated region. The relevant fragment was added to an agarose gel and purified using a QIAquick gel extraction kit (QIAGEN Inc.). The MfeI/Msel restriction fragment was ligated into the backbones of plasmid pMG20 and pBM23, which 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 site digestion.

Studies of the Effects of pH, Ionic Strength, and Temperature on the Optical Properties of the A264E Heme Domain

UV-visible spectra were recorded for the substrate-free form of the A264E BM3 enzymes and heme domain in 50 mM potassium phosphate (pH 5.0–9.0) at 0.5 pH unit intervals. Spectral perturbations were observed, and absorption data reflecting the maximum overall change between the low- and high-pH spectra for P450 BM3 and A264E (with reference to the spectrum collected at pH 7.0) were plotted against pH and fitted to a sigmoid to derive an apparent pH midpoint for absorption. Further UV-visible spectra for the substrate-free form of the A264E BM3 enzymes and heme domain (4 μM) were collected in 20 mM MOPS (pH 7.4) and in the same buffer containing potassium chloride at 0–1 M. Spectra were recorded for the A264E heme domain (4 μM) in assay buffer at temperatures between 18 and 66 °C in 2 °C 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 system. A 2-min equilibration time was allowed at each temperature point in the titration prior to collection of the spectrum.

The abbreviations used were: MOPS, 4-morpholinopropansulfonic acid; MCD, magnetic circular dichroism; CT, charge-transfer.
Redox Potentiometry

All redox titrations were carried out in an anaerobic glove box (Belle Technology, Portesham, United Kingdom) under a nitrogen atmosphere, with oxygen levels maintained at < 5 ppm. Redox titrations were carried out for both the wild-type and A264E P450 BM3 heme domains (typically 6–10 μM) in an anaerobic solution in 1% deuterium oxide and connected to a Cary UV-50 Bio UV-visible spectrophotometer (Varian Instruments) outside the glove box. Potentials were measured using a Hana pH 211 meter coupled to a platinum/calomel electrode (ThermoRussell Ltd.) at 25 ± 2 °C. The electrode was calibrated using the Fe3+/Fe2+-EDTA couple as a standard (+108 mV). A factor of +244 mV was used for correction relative to the standard hydrogen electrode. Mediators were added to facilitate electrical communication between electrode and solution. Typically, 2 μM phenasine methosulfate, 5 μM 2-hydroxy-1,4-naphthoquinone, 0.5 μM methyl viologen, and 1 μM benzyliol were included to mediate in the range between +100 and −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 analyzed by plotting the absorbance at an appropriate wavelength, corresponding to the maximum absorbance change between oxidized and reduced forms, against the potential. A 1-electron Nernst function was then fitted to the data to describe the transition for the Fe3+/Fe2+-EDTA couple as a standard (+108 mV). A factor of +244 mV was used for correction relative to the standard hydrogen electrode. Mediators were added to facilitate electrical communication between electrode and solution. Typically, 2 μM phenasine methosulfate, 5 μM 2-hydroxy-1,4-naphthoquinone, 0.5 μM methyl viologen, and 1 μM benzyliol were included to mediate in the range between +100 and −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.

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Novel Heme Ligation in a Cytochrome P450

UV-visible spectroscopy indicated that the oxidized form of the pure enzymes incorporated heme fully and had Soret/A<sub>405</sub> ratios very similar to those of 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 with 3,3',5,5'-tetramethylbenzidine following the methods described by Thomas et al. (32). In contrast to the results of Betti and co-workers (23, 26) and of Ortiz de Montellano et al. (24, 25) with various CYP4 isoforms, there was no discernible staining of the P450 BM3 heme with 3,3',5,5'-tetramethylbenzidine that would indicate the presence of covalently bound heme. In view of previous results indicating turnover-dependent formation of the covalent link- age in CYP4 isoforms, the A264E heme domain and full-length flavocytochrome were preincubated both with and without excess arachidonic acid in the presence or absence of excess reductant (dithionite or NADPH). Additional samples (in the presence or absence of arachidonate) were exposed to hydrogen peroxide (which can drive P450 catalysis via the "peroxide shunt" pathway). All samples were incubated at ambient temperature or at 30 °C for periods between 5 and 30 min for reactions to occur. Samples 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 c and S. frigidimaris flavocytochrome c<sub>3</sub>. Both of these enzymes contain covalently attached 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 b-type heme did not remain bound to the proteins in either the wild-type or A264E forms.

UV-visible Spectroscopy—Despite the apparent lack of cova lent 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 the oxidized substrate-free A264E flavocytochrome and heme domain was shifted to a longer wavelength by -2 nm with respect to the wild-type forms (from -418 to 420.5 nm), and there were similar small perturbations in the a(3)/b-band region (from -568 to 571 nm for the a-band and from 534 to 538 nm for the b-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 a/β-bands apparently fused with a maximum at 546 nm (Fig. 2A). The spectral properties of the ferrous-carbon monoxide complexes were also determined, showing a Soret shift to -449 nm for the A264E heme domain compared with -448 nm for the wild-type enzyme (30, 40). To analyze further the spectral properties of the A264E mutant, a nitrosyl complex was generated by bubbling the A264E heme domain with NO gas (five small bubbles). A Soret band shift to 435 nm was observed (compared with 434 nm for the wild-type P450 BM3 heme domain). As with the wild-type enzyme, there was a marked increase in intensity of the α- and β-bands, with their maxima shifted to 575 and 543.5 nm, respectively. The binding of a tight-binding azoee 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 a/β-band intensity and shifts to 575/544 nm (Fig. 2B).

To examine the 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 upon the addition of fatty acids to A264E were distinct from those observed previously for wild-type P450 BM3 and various mutant forms (see Ref. 31). Neither lauric acid nor myristic acid (C<sub>12</sub> and C<sub>14</sub> saturated fatty acids, respectively) induced any significant change in the spectral properties of the A264E mutant, even at concentrations near their solubility limits (950 and 250 μM, respectively). By contrast, the longer chain fatty acids palmitic acid (C<sub>16</sub> saturated), palmitoleic acid (C<sub>16</sub> monoensaturated), and arachidonic acid (C<sub>20</sub> polyunsaturated) all induced spectral changes. However, rather than inducing a type I optical change with a shift in the Soret maximum to a shorter wavelength, a Soret shift to a final wavelength maximum of ~426 nm was observed in all cases. The final extent of the spectral shift induced was dependent on the particular fatty acid used, with arachidonic acid being the
I showing the substrate-free A264E heme domain showing progressive deviation from the starting spectrum. The solid line, arachidonic acid to the A264E heme domain.

ence spectra heme domain generated by subtraction of the starting absolute spectrum. The final spectrum shown was recorded at 8.25 following the addition of 1.65, 2.97, and 4.95 μM arachidonic acid at intervals. Selected difference spectra (with progressive labels) indicate reinforcement of the low-spin form of the cytochrome.

Data for the optical titration with arachidonic acid are shown in Fig. 3. As the pH was lowered, there was a consistent shift in the absorption maximum of the Soret band toward shorter wavelengths, 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 ionizable group and possibly reflecting the pKₐ value for a protonatable amino acid side chain. The apparent pKₐ 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 assay buffer and in the same MOPS buffer with KCl at 0–1 mM intervals. Increasing the ionic strength produced negligible effects on the spectrum of the 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 in the Soret band to a longer wavelength. In 20 mM MOPS (pH 7.4), the A264E Soret band was centered at ~419 nm, moving to 420.5 nm in the typical 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 produced type II optical shifts in the A264E heme domain (and intact flavocytochrome) that are comparable with those induced by the addition of long-chain fatty acids to the mutant and that are indicative of a novel ligation state of the heme iron.

Steady-state Kinetic Parameters—The kinetic properties of the wild-type and A264E flavocytochrome P450 BM3 enzymes were determined with respect to their capacity to catalyze 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, the kinetics were studied in assay buffer, which we have used in several previous studies of P450 BM3. As expected, the A264E mutant catalyzed rapid NADPH-dependent reduction of cytochrome c (mediated via the FMN domain of the enzyme), indicating that the difluor reductase domain of the enzyme is catalytically unimpaired by the mutation in the heme domain. However, the rates of fatty acid-dependent NADPH oxidation were considerably slower than those for wild-type P450 BM3 (Table 1).

The most effective among those tested. This aspect of the mutant’s behavior is discussed in more detail under “Discussion.” Thus, rather than undergoing a substrate-dependent optical transition typical of increased high-spin heme iron content, the A264E mutant showed instead a type II transition usually observed upon ligation of inhibitors to the heme iron (e.g., imidazoles) (Fig. 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 Fig. 3.

Notwithstanding the unusual spectral changes observed, the apparent binding constants (Kₐ) were determinable from plots of induced spectral changes versus fatty acid concentration as described under “Experimental Procedures.” The Kₐ 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).

Although myristic acid and lauric acid failed to produce spectral changes of sufficient magnitude to facilitate any accurate determination of their apparent Kₐ values for the A264E heme domain, the same fatty acids did 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 was lower than that produced with the other fatty acids tested here. It should also be noted that the apparent Kₐ values determined for palmitate, palmitoleate, and arachidonate with the A264E heme domain are all considerably tighter than those for the wild-type heme domain (Table 1).

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 of 5.0 to 9.5. In the interval 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 (Fig. 2). As the pH was lowered, there was a consistent shift in the absorption maximum of the Soret band toward shorter wavelengths, 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 ionizable group and possibly reflecting the pKₐ value for a protonatable amino acid side chain. The apparent pKₐ 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 assay buffer and in the same MOPS buffer with KCl at 0–1 mM in 100 mM intervals. Increasing the ionic strength produced negligible effects on the spectrum of the 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 in the Soret band to a longer wavelength. In 20 mM MOPS (pH 7.4), the A264E Soret band was centered at ~419 nm, moving to 420.5 nm in the typical 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 produced type II optical shifts in the A264E heme domain (and intact flavocytochrome) that are comparable with those induced by the addition of long-chain fatty acids to the mutant and that are indicative of a novel ligation state of the heme iron.

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TABLE I

<table>
<thead>
<tr>
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<th>Wild-type BM3</th>
<th>A264E BM3</th>
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<tbody>
<tr>
<td></td>
<td>K&lt;sub&gt;d&lt;/sub&gt;</td>
<td>k&lt;sub&gt;a&lt;/sub&gt;</td>
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<tr>
<td>Laurate</td>
<td>100 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2770 ± 120</td>
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<tr>
<td>Myristate</td>
<td>6.9 ± 0.4</td>
<td>4835 ± 295</td>
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<tr>
<td>Palmitate</td>
<td>11.3 ± 0.4</td>
<td>4590 ± 407</td>
</tr>
<tr>
<td>Arachidonate</td>
<td>3.5 ± 0.17</td>
<td>17100 ± 190&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Palmitoleate</td>
<td>0.55 ± 0.05&lt;sup&gt;e&lt;/sup&gt;</td>
<td>6980 ± 430</td>
</tr>
<tr>
<td>Cytochrome e</td>
<td>11050 ± 630</td>
<td>16.1 ± 4.1</td>
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<tr>
<td>4-Phenylimidazole</td>
<td>0.85 ± 0.45&lt;sup&gt;e&lt;/sup&gt;</td>
<td>16.1 ± 4.1</td>
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<sup>a</sup> Data are from Noble et al. (71).
<sup>b</sup> Data were fitted to Equation 1.
<sup>c</sup> Data are from Noble et al. (31).

The comparative kinetic (k<sub>a</sub>/K<sub>i</sub>) and substrate/inhibitor binding (K<sub>i</sub>) constants for the 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 domains. Kinetic constants were derived from steady-state assays with the wild-type and A264E full-length flavocytochromes. All data were collected as described under "Experimental Procedures." Data were fitted to rectangular hyperbolae, except where indicated. Selected values of k<sub>a</sub>/K<sub>i</sub> and K<sub>i</sub> for the wild-type enzyme from previous studies are indicated.

Absence of fatty acids, both the wild-type and A264E flavocytochrome P450 BM3 enzymes oxidized NADPH at a slow rate (~5 min<sup>-1</sup>). In the presence of either lauric acid or myristic acid, there was considerable stimulation of NADPH oxidase activity, despite the fact that the 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 I). Thus, it appears that A264E flavocytochrome P450 BM3 retains considerable levels of activity with long-chain fatty acids, despite the fact that these fatty acids induce either negligible change toward the high-spin state or inhibitor-like optical change to the heme spectrum.

**Fatty Acid Oxidation**—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 is linked to oxygenation of myristic acid, palmitic acid, and palmitoleic acid. Turnover studies were performed as described under "Experimental Procedures," and products were examined by mass spectrometry. Products were evident from turnover of each of these substrates. In the case of myristic acid, there were similar levels of production of monooxygenated product for both the wild-type and A264E flavocytochrome P450 BM3 enzymes. With palmitic acid, mass spectrometry showed the presence of both mono- and dioxygenated products for both the 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 (11, 41). With the monounsaturated fatty acid palmitoleic acid (cis-9-hexadecenoic acid) as the substrate, both mono- and dioxygenated products were observed for both the wild-type and A264E enzymes, with rather lower amounts of products with the mutant enzyme. In previous studies, Fulco and co-workers (42, 43) demonstrated that both hydroxylation (close to the ω-terminal) and epoxidation (across the C<sub>9</sub>–C<sub>10</sub> double bond of palmitoleic acid) are catalyzed by wild-type P450 BM3. Under the same experimental conditions, the amounts of products generated from palmitic acid and palmitoleic acid by the A264E mutant were ~30–40% lower than those produced by wild-type P450 BM3, consistent with the differences in steady-state kinetics shown in Table I. 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 wild-type P450 BM3 (44). Thus, despite the unusual spectral conversions produced upon 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 compared with that seen in the wild-type enzyme and that substrate addition, basic pH, and high ionic strength induce optical changes indicative of a change in the heme iron coordination state. Although no detectable covalent ligation of the heme macrocycle was detectable with A264E flavocytochrome P450 BM3, it appeared likely that the glutamate may instead interact with the heme macrocycle or, more likely, coordinate to the heme itself to produce the spectral perturbations observed.

For this reason, we undertook spectroscopic analysis to examine structural features of the mutant protein and its bound heme cofactor.

**Circular Dichroism**—The 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 α-helical A264E heme domain has a far-UV CD spectrum virtually identical to that of the wild-type enzyme, with minima at 223 and 209 nm and an absorbance at 202 nm. The far-UV CD spectrum for A264E (as with the wild-type enzyme) was not significantly altered by the addition of arachidonic acid, showing that secondary structural content is not significantly altered upon the binding of 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 of the 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 centered at ~400 nm and shifts to ~404 nm upon arachidonic acid binding (Fig. 4). Thus, the direction of movement of the Soret band (to shorter wavelengths) 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. Upon the addition of arachidonic acid, this feature sharpened and shifted to 426 nm, the same wavelength seen for the Soret maximum in the optical spectrum of the arachidonate-bound A264E heme domain (Fig. 4). Thus, near-UV-visible CD spectroscopy shows much more marked differences in the spectra of the wild-type and A264E heme enzymes than does optical absorption spectroscopy. A large spectral difference is seen between the near-UV-visible CD spectra of the substrate-free forms of the wild-type and A264E heme.
domains, whereas (under identical conditions) optical absorption shows a shift of only ~1.5 nm in the Soret band.

**Resonance Raman Spectroscopy—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 were observed upon the addition of fatty acids, including shifts in position and intensity of the spin-state marker bands $v_2$ and $v_1$, reflecting the change in heme iron spin-state equilibrium toward the high-spin form (30). By contrast, there were no significant changes in any of the oxidation (e.g. intensity of $v_2$) remained constant at 1371 cm$^{-1}$) or spin-state (e.g. intensity of $v_1$) remained constant at 1501 cm$^{-1}$) marker bands for the A264E heme domain following the addition of arachidonic acid. Resonance Raman spectroscopy confirmed that the substrate-free enzyme is essentially completely low-spin and that the addition of arachidonate reinforces the low-spin state. The only significant differences following fatty acid addition to the A264E heme domain were small increases in intensity of the $v_1$ and $v_2$ band at 1621 cm$^{-1}$, relating to the heme vinyl groups, and of the $v_1$ band at 1560 cm$^{-1}$. The $v_1$ 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 spectroscopy did not provide specific vibrational information on the proposed switch of distal ligands to the heme iron that occurred upon arachidonic acid binding to the A264E heme domain. For such information, we turned instead to EPR and MCD spectroscopies.

**EPR Spectroscopy—**EPR spectra of the arachidonate-bound and substrate-free forms of the wild-type and A264E heme domains were recorded and are shown in Fig. 5A. The EPR spectrum for wild-type P450 BM3 is as previously reported (45) and is typical for low-spin ferric P450 enzymes, which all give rise to spectra with $g$ in the range 2.40–2.45 (46–49). Upon the binding of substrate, the ferric spin equilibrium was perturbed, and the iron became a mixture of low- and high-spin forms. The latter appears in the EPR spectra with features at $g = 8.18, 3.44, and 1.66$, which originate in the lowest ($m_s = \pm 1/2$) 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 ($D \gg gH$), these values correspond to a rhombicity of $E/D = 0.11$. In ferric hemo-
teins, such substantial rhombicities are found only with thiolate ligation. The EPR spectra of the substrate-free and arachidonate-bound A264E P450 BM3 enzymes suggest the presence of several low-spin ferric species, all with g-values that indicate that thiolate coordination has been 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 of $>2.45$. The $g_z$ value of $\sim 2.56$ must therefore indicate ligation different from that of the wild-type enzyme and is strong evidence for coordination of the distal glutamate. In support of this, the formate derivatives of P450cam and chloroperoxidase give $g_z = 2.55$ and 2.59, respectively (49, 50). 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 subpopulation. In contrast to the wild-type P450 BM3 enzyme, when substrate was bound to the A264E mutant, there was 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 O$_2$. A comparison of the EPR spectra for the 4-phenylimidazole-bound forms of the wild-type and A264E heme domains is shown in Fig. 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 signal is broadened, suggesting that there may be a split population of two conformers with a slightly different g component and paired with the two subtly different g components. Minor signals at $g_z \sim 2.44$ and 1.92 may reflect a small proportion of unligated enzyme. In the azole-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 the residual aqua-ligated components seen in the wild-type enzyme spectrum). However, it appears that a single conformational form of azole-bound heme is present in A264E, whereas there may be two distinct species in wild-type P450.

**MCD**—The MCD spectra for the wild-type P450 BM3 heme domain are consistent with those we have reported previously (45). The room temperature near-UV-visible MCD spectra of the substrate-free wild-type and A264E P450 BM3 enzymes and the arachidonate-bound A264E enzyme (Fig. 6A) each show a pattern of bands typical for low-spin ferric hemes with a thiolate ligand. The unusually low MCD intensity in the Soret band (400-420 nm) and the $\alpha$-region (500-600 nm) is also characteristic of such species (47, 51-59). A small additional negative feature at $\sim 655$ nm is part of a derivative-shaped negative $\beta$-band and is only discernible at a low level (115%) of the high-spin form (see Ref. 54). 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 charge-transfer (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 $\sim 555$ 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 spectra and has been located in the room temperature near-infrared MCD spectra for wild-type P450 BM3 minus substrate and for the A264E mutant in both the absence and presence of substrate at $\sim 1080$ nm (Fig. 6B), as was previously reported for wild-type P450 BM3 at low temperature (45). Close inspection of these three spectra reveals qualitative and quantitative differences. The peak position for the transition shifts from $\sim 1075$ nm for the substrate-free wild-type enzyme through to $\sim 1080$ nm for the substrate-bound A264E mutant and to $\sim 1085$ nm for the arachidonate-bound A264E mutant. Differences in both the breadth and intensity of the CT band are discernible (Fig. 6B). The exact energy of this CT transition is generally diagnostic of the two heme axial ligands (60, 61), 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 nitrogenuous ligand distal to cysteinate, the CT transitions are at 1180 nm (45), 1120 nm (60), and 1150 nm (62), respectively.

The addition of arachidonic acid substrate to wild-type P450 BM3 resulted in a marked change in the near-infrared 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 was significantly diminished. The derivative-shaped MCD band centered at $\sim 900$ nm is the CT band characteristic of high-spin ferric heme and is ex-
tremely similar to that reported for substrate-bound cytochrome P450cam (63).

**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 toward the high-spin form (21, 30). In P450 BM3 (as in P450cam), this is accompanied by a change in the heme iron reduction potential of ~130–140 mV (from ~−427 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 the addition of excess dithionite. By contrast, it was difficult to reduce the substrate-free wild-type heme domain completely using dithionite and impossible under aerobic conditions because of its negative potential and rapid reoxidation of the ferrous form. Upon the addition of near-saturating arachidonate, the A264E Soret band was located at ~426 nm and shifted to a final position of ~410 nm upon complete reduction of the heme iron during the redox titration (Fig. 7A). In the substrate-free form, the Soret band of the oxidized A264E heme domain was located at 419.5 nm and shifted progressively to a final position of ~410 nm upon complete reduction of the heme iron (Fig. 7A). Absorption versus potential data were plotted at 410 nm and were fitted to a 1-electron Nernst equation to define the midpoint potential for the A264E heme iron in the presence and absence of arachidonate. These values were ~−318 ± 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.

**DISCUSSION**

The capacity of the eukaryotic family 4 cytochrome P450 enzymes to link their heme macrocycle covalently to the protein backbone has been one of the most significant discoveries in P450 research in recent years (see Refs. 23 and 24). From a biotechnological perspective, the ability to covalently link the porphyrin to the P450 protein matrix is attractive for at least two reasons. First, the presence of the glutamate and the glutamate ligation process were shown to enhance catalytic activity in rabbit CYP4B1 (26), and enhancement of catalytic rate is clearly a desirable feature to endow an enzyme. Second (and the major reason), the capacity of P450 enzymes to undergo conversion to the inactive P420 form (in which native cytochrome heme ligation is lost) is well recognized, and heme can even be dissociated completely from the P450 enzyme under moderately denaturing conditions (see Ref. 16). Thus, the ability to covalently link heme should promote longevity of P450 activity, particularly since the P450- to-P420 conversion has been shown to be reversible in selected P450 enzymes, including P450 BM3 (see Ref. 19). From a perspective of exploitation of P450 enzymes, the bacterial enzymes P450cam and P450 BM3 have been the most intensively studied. Rational mutagenesis of P450cam has produced variants of the camphor hydroxylase that are able to oxygenate molecules such as butane and propane (64). Rational mutagenesis of the fatty-acid hydroxylase P450 BM3 has produced variants that catalyze oxygenation of fatty acids at different positions compared with the wild-type form and in which substrate selectivity has been converted toward short-chain fatty acids and polyaromatic hydrocarbons such as phenanthrene (21, 28). 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 functionalized hydrocarbons. In view of this potential and the close relationship between P450 BM3 and the eukaryotic family 4 fatty-acid hydroxylases, we mutagenized P450 BM3 to introduce the glutamate conserved in the eukaryotic family 4 P450 enzymes at the same helix position in P450 BM3 (i.e. Ala^264) 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 the substrate-free and palmitoleate-bound forms (see accompanying article (72)).

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 for the substrate-free form of A264E enzyme (Soret band at 420.5 nm compared with 419 nm for 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 toward the glutamate-ligated form in the presence of long-chain fatty acids (the preferred substrates for P450 BM3). Increasing ionic strength and basic pH had a similar effect on the optical spectra; and in the latter case, an apparent pH 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 and lauric acid) had an 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 (65) determined the atomic structure of oxidized palmitoleate-bound wild-type P450 BM3 and highlighted the interaction of the carboxylate group with the Tyr49/Arg47 motif near the mouth of the substrate entry channel. The o-terminals of the longer chain fatty acids will extend further toward 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 Phe67) with the glutamate and to induce its repositioning to interact with the heme iron. The shorter chain fatty acids, with their carboxylate still tethered at the active-site mouth, are predicted not to extend far enough toward the heme to enable interaction with the glutamate. Again, these predictions are supported by structural data (see accompanying article (72)).

Spectroscopic studies were undertaken to provide further proof that a novel form of heme iron ligation occurred in the A264E P450 BM3 variant. Resonance Raman spectroscopy provided data consistent with those obtained from optical titrations, confirming that the low-spin form is reinforced upon addition of substrate. However, it could not provide convincing data regarding the proposed switch to Cys-Glu coordination. EPR and MCD spectroscopies could provide such data. EPR studies of the 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 toward 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 is consistent with the structure of the palmitoleate-bound form of the enzyme. The EPR spectrum for the glu-ligated form(s) of P450 BM3 are consistent with previous studies of P450cam in complex with oxygen donor ligands (e.g. formate, acetate, and propionate), and there is particularly strong similarity to formate-bound P450cam, which displays g-values at 2.55, 2.25, and 1.88 (49, 52).

A limited number of heme iron ligand sets are observed in natural cytochromes. These include His-Met (e.g. in cytochrome cd, from Pseudomonas aeruginosa), Met-Met (bacterioferritin from the same bacterium), and His-His (e.g. eukaryotic cytochrome b5) (67–69). Cysteinate-ligated heme enzymes include the Cys-aqua-ligated cytochrome P450 enzymes and the nitric-oxide synthases (see Refs. 45 and 49), and the Cys-His-ligated heme in the SoxAX protein from Rhodovulum sulfidophilum, with a role in thiosulfate oxidation (60). 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, the binding of long-chain fatty acids forces the equilibrium heavily in favor of the novel Cys-Glu ligand set in a similar fashion as the binding of these substrates induces aqua ligand displacement and formation of a high-spin five-coordinate heme iron species in 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 toward 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 the tightly bound fatty acid substrate to be liberated. In reporting the substrate subsequent to heme iron reduction (70). Thus, it appears clear that the Cys-Glu coordination is broken following heme iron reduction, enabling binding of oxygen and catalysis to ensue, albeit with lower catalytic rate than in the wild-type enzyme. Potentiometric analysis (Fig. 7) demonstrated that the reduction potentials of the sub-
state-bound and substrate-free forms of the A264E heme domain are similar to one another (−316 ± 5 mV) and to that of the fatty acid-bound wild-type enzyme (−289 mV), consistent with the capacity of the reductase domain to mediate electron transfer to the 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 archaichrome-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 toward the former as the reductive titration progresses. The altered heme iron reduction potential of the A264E mutant is discussed further in the accompanying article (72) 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. The structural reasons underlying this substrate-induced equilibrium perturbation are described in the accompanying article (72). Differences in active-site architecture between P450 BM3 and the mammalian CYP4 enzymes likely explain the failure of the A264E P450 BM3 variant to covalently link the heme macrocycle to the glutamate side chain. 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 Aln244 could generate additional sets of proteinaceous heme ligands that have not yet been observed in nature, possibly prompting the discovery of such cytochromes. This offers exciting possibilities for structural and spectroscopic study. In ongoing work, we are generating a series of Aln244 mutants in P450 BM3 in attempts to produce and characterize additional new heme iron coordination states.

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REFERENCES

Novel Heme Ligation in a Cytochrome P450

A Single Mutation in Cytochrome P450 BM3 Induces the Conformational Rearrangement Seen upon Substrate Binding in the Wild-type Enzyme*

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The multidomain fatty-acid hydroxylase flavocytochrome P450 BM3 has been studied as a paradigm model for eukaryotic microsomal P450 enzymes because of its homology to eukaryotic family 4 P450 enzymes and its use of a eukaryotic-like flavin reductase redox partner. High-resolution crystal structures have led to the proposal that substrate-induced conformational changes lead to removal of water as the sixth ligand to the heme iron. Concomitant changes in the heme iron spin state and heme iron reduction potential help to trigger electron transfer from the reductase and to initiate catalysis. Surprisingly, the crystal structure of the substrate-free A264E heme domain mutant reveals the enzyme to be in the conformation observed for substrate-bound wild-type P450, but with the iron in the low-spin state. This provides strong evidence that the spin-state shift observed upon substrate binding in wild-type P450 BM3 not only is caused indirectly by structural changes in the protein, but is a direct consequence of the presence of the substrate itself, similar to what has been observed for P450cam. The crystal structure of the palmitoleate-bound A264E mutant reveals that substrate binding promotes heme ligation by Glu284, with little other difference from the palmitoleate-bound wild-type structure observable. Despite having a protein-derived sixth heme ligand in the substrate-bound form, the A264E mutant is catalytically active, providing further indication for structural rearrangement of the active site upon reduction of the heme iron, including displacement of the glutamate ligand to allow binding of dioxygen.

Cytochrome P450 enzymes are among the most studied enzymes, in no small part because of the pivotal roles that hepatic P450 enzymes play in mammalian drug metabolism (1). Recent years have seen an explosion in the structural data available for these systems, a substantial proportion of which is on cytochrome P450 BM3 (2). This multidomain enzyme is isolated from Bacillus megaterium and contains an N-terminal fatty acid-binding P450 domain fused to its redox partner, an NADPH-dependent flavin-cytochrome P450 reductase (3). It has been used as the paradigm model for studying the similar, but membrane-associated eukaryotic microsomal P450 systems. This has been primarily because its heme domain is similar to the eukaryotic fatty-acid hydroxylases from P450 family 4, because the enzyme is soluble and uses a eukaryote-like cytochrome P450 reductase as the redox partner (as opposed to the two-component ferredoxin reductase and ferredoxin systems found in many other bacterial systems), and because it is a convenient catalytically sufficient fusion protein enzyme (2–4).

Cytochrome P450 BM3 is a fatty-acid hydroxylase that displays an unusually high rate of oxygenation of long-chain fatty acids (e.g. >15,000 turnovers/min with arachidonic acid) (5), likely because of the efficient electron transfer between the different redox modules afforded by their covalent linkage and hence close spatial organization (2). A sophisticated mechanism to avoid the unwanted generation of reactive oxygen species through futile cycling has been found in many P450 enzymes studied to date. The binding of oxygen occurs only with the reduced (ferrous) heme, and the reduction of the ferric heme iron by electron transfer from the redox partner is, in turn, dependent on the binding of substrate, effectively gating initiation of the reaction by substrate binding (6, 7). In P450 BM3 and the Pseudomonas putida camphor hydroxylase P450cam (the most intensively studied P450 enzyme), substrate binding induces a heme iron spin-state shift and a concomitant increase in the reduction potential of the heme iron, favoring the 1-electron reduction that commits the enzyme to the catalytic cycle (6, 7). The molecular mechanism whereby substrate binding induces this shift seems to be somewhat different in the P450 enzymes studied to date, although the substrate binding-induced displacement of water as the sixth ligand to the heme iron is a common feature (see Refs. 8 and 9). In P450cam, the binding of substrate does not affect any large-scale changes in the protein structure, and the displacement of water is due to direct steric hindrance with the camphor molecule (10). In contrast, P450 BM3 undergoes large-scale conformational changes upon binding of fatty acids, and these changes have been proposed to drive the conversion of a six- to a five-coordinate heme group. Indeed, there is no direct interaction observed between bound fatty acids and the water molecule in the sixth ligand position in the available crystal structures of the P450 BM3 heme domain. The conformational change in P450 BM3 involves a reorganization of the I helix, and it has been proposed that this creates a new water binding position (11).

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This position is mutually exclusive with the sixth ligand binding position and is proposed to have greater affinity, leading to an effective switch in heme coordination by transfer of the water to the new position. The majority of the P450 structures indeed show a conserved bend in the I helix, and this mechanism might therefore be of a general nature (see Ref. 12). However, certain P450 enzymes do not contain any deformation of the I helix in the resting state (see Ref. 13), whereas P450cam has a bent I helix that does not significantly change conformation upon substrate binding (14).

We present here the crystal structures of both the substrate-free and palmitoleic acid-bound forms of the A264E mutant in the P450 BM3 heme domain. The alanine occupies a key position in the I helix of the P450 enzyme. The backbone of this residue is in the I helix of the P450 enzyme. The backbone of this residue is substantially shifted upon substrate binding (14). Interestingly, in several of the CYP4 family of fatty-acid oxygenase P450 enzymes, a conserved glutamate residue at position 264 (BM3 numbering) is known to covalently ligate the heme macrocycle through autocatalytic turnover-dependent attachment to the 5-methyl group of the porphyrin (16). To investigate the possibility of creating a similar protein-heme link in the related P450 BM3 heme domain, the A264E mutant was created. Although no covalent modification of the heme was observed, this mutant has several unique features (see accompanying article (17)). In the fatty acid-free form, Glu264 ligates the heme iron in a proportion of the molecules, creating a novel thiolate-carboxylate ligation that is pushed toward full ligation by binding of the substrate. Surprisingly, the crystal structure of the substrate-free form of this mutant reveals the protein to be in the conformation previously considered to be induced by substrate binding, despite the fact that the P450 enzyme is still in a low-spin state and free of fatty acid. We show that binding of fatty acid does not then introduce any further gross conformational change in the protein structure, although a change in the proportion of molecules in which glutamate ligates the heme iron is effected. The implications of these observations for the mechanism of the substrate-binding-induced heme iron spin-state shift of P450 BM3 and P450 enzymes in general are discussed, along with the ramifications for understanding conformational equilibria in P450 enzymes and how the binding of substrate impacts on these equilibria.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis, Expression, and Purification of Mutant P450 BM3**—The A264E mutant of the P450 BM3 heme domain was created, expressed, and purified as described in the accompanying article (17). A final purification step by fast protein liquid chromatography using Q-Sepharose resin (under the same conditions as used for the low-pressure chromatographic purification described in the accompanying article (171)) was used to produce homogeneous enzyme for crystallographic studies.

**Crystalization**—The P450 BM3 A264E heme domain was crystallized in both the palmitoleic acid-bound and substrate-free forms by the sitting drop method at 4 °C. Sitting drops were prepared by adding 2 μl of mother liquor to 2 μl of 15 mg/ml enzyme. Palmitoleic acid-bound crystals were obtained by co-crystallizing the enzyme with 1.2 μl palmitoleic acid (~6 times the Kd value obtained from spectral binding titration data) and a well solution of 100 mM magnesium acetate, 20% polyethylene glycol 2000MME, and 10 mM cacodylic acid at pH 6.3. Substrate-free crystals were obtained with a well solution of 10 mM magnesium sulfate, 20% polyethylene glycol 2000MME, and 10 mM cacodylic acid at pH 6.3. Crystals were formulated in both cases after ~7 days. Crystals were immersed in 10% polyethylene glycol 200 in mother liquor as a cryoprotectant, before being mounted on a nylon loop and flash-cooled in liquid nitrogen.

**Data Collection, Structure Elucidation, and Refinement**—The data used for refinement were collected at the European Synchrotron Radiation Facility (Grenoble, France) on ID14-EH1 using an ADSC Q4R CCD detector. Crystals were cooled at 100 K, and diffraction data were collected in oscillations of 1°. Data were processed and scaled using the HKL program package DENOZO and SCALPACK (18). The substrate-free crystal structures were solved via molecular replacement using the program AMORE and the high-resolution wild-type P450 BM3 crystal structure (Protein Data Bank code 2HPD) (19) as a search model. The palmitoleate-bound wild-type crystal structure was used a starting model for the palmitoleate-bound mutant form. In both cases, positional and B-factor refinement was carried out using REFMAC5 with manual rebuilding of the model at regular intervals in TURBIO-FRODO (20, 21). Only in the case of the low-resolution substrate-bound crystal form were strong NCS restraints imposed throughout refinement. Data collection and final refinement statistics are given in Table I. The atomic coordinates and structure factors for both crystal structures have been deposited in the Protein Data Bank with codes 1SMJ (substrate-free A264E mutant) and 1SMJ (substrate-bound A264E mutant).

**RESULTS AND DISCUSSION**

**Crystal Structure of the Substrate-free Form**—Initial crystalization trials for the substrate-free form of the A264E heme domain of P450 BM3 using the published conditions for the wild-type heme domain proved unsuccessful. The use of magnesium sulfate in place of magnesium acetate led to related conditions that generated large diffraction-quality crystals. The structure was solved to 2.0 Å and contained two molecules in the asymmetric unit. The overall conformation of both molecules is similar (root mean square deviation of 0.46 Å for all Cα atoms), with molecule B having a significantly higher

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**Table I Crystallographic statistics**

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<th>Protein</th>
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<td>15.0–2.75</td>
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*ESRF, European Synchrotron Radiation Facility; r.m.s., root mean square.*
average B-factor (39.1 Å² compared with 47.6 Å²) due to fewer packing constraints. In this study, molecule A will be used for discussion and calculations unless mentioned otherwise.

Because of the different space groups of the orthorhombic A264E mutant and the wild-type monoclinic crystal form, comparison of the structures was made after overlay using the structurally invariant residues, as described by Haines et al. (11), representing ~62% of the structure. Surprisingly, it was found that the A264E structure is structurally similar (root mean square deviation of 0.54 Å for all Cα atoms) to the substrate-bound form of the wild-type enzyme (hereafter referred to as the SB1 conformation) and shows a significant difference (root mean square deviation 1.37 Å for all Cα atoms) from the substrate-free wild-type structure (hereafter referred to as the SF conformation) (Fig. 1, upper and lower) (8, 15).

However, no substrate was added to the A264E mutant during either purification or crystallization, and no substrate could be observed in the electron density maps. Similar to the changes seen upon substrate binding in wild-type P450 BM3, the majority of residues that are in significantly different positions between the substrate-free structures of A264E and the wild-type heme domain are located in the “lid domain” of the substrate access channel, which consists of the F and G helices, the loop between them, and the D′ helix (8). Several of these residues are less well defined in A264E molecule B, indicating substantial plasticity in this region in the absence of substrate. However, there is no significant large-scale difference between both A264E molecules in the asymmetric unit.

In contrast, upon closer inspection of the active site of the A264E enzyme, there is a marked difference in the vicinity of the heme iron between the two molecules in the asymmetric unit. Molecule B has the side chain of Glu264 pointing away from the heme, its carboxylate group stacking with the aromatic group of Phe87 (Fig. 2, left). In contrast, molecule A has the carboxylate group coordinating the heme iron (Fig. 2, right). This heterogeneity of glutamate ligation was predicted from solution spectrophotometric studies and from EPR analysis (see accompanying article (17)), and the thiolate-carboxylate ligation is novel to P450 enzymes and (as far as we are aware to date) to cytochromes in general. There do not seem to be any significant conformational changes to the overall P450 structure associated with the “switch” of Glu264 between its two detected conformations on (a) the heme iron or (b) stacking with Phe87. It is likely that, in solution, the carboxylate continues to switch between the “on” and “off” heme iron states without major accompanying protein reorganization. According to spectroscopic data, in the solution state, the equilibrium is poised at ~3–4:1 in favor of the heme iron ligand off form (17). The interaction with Phe87 is particularly interesting, given the fact that this residue is absent from the CYP4 enzymes in which covalent ligation of the heme methyl group has been demonstrated. Phe87 interacts with the α-methyl group of fatty acid substrate(s) in wild-type P450 BM3 and is considered to be a critical regulatory residue that controls regioselectivity of substrate oxygenation (8, 22, 23). A particular difference in the behavior of P450 BM3 with respect to eukaryotic CYP4 enzymes is the inability of the former to hydroxylate at the α-position (24).

Close examination of the A264E structure reveals no clear direct structural explanation for the fact that this mutant mimics the conformation of the substrate-bound form of the wild-type enzyme. In particular, no extra stabilizing features involving the newly introduced glutamate side chain can be found in comparison with available wild-type structures. We therefore tried to place the Glu264 side chain in the corresponding substrate-free wild-type structure. All of the conformations available to the glutamate result in severe steric clashes with several other residues nearby (e.g. Phe87, Thr260, and His263, in
addition to the heme macrocycle itself), clearly resulting in the need for a protein conformational rearrangement to accommodate for the increased bulk of residue 264. It therefore seems likely that the A264E mutation does not particularly stabilize the SB conformation, but rather destabilizes the SF conformation of the enzyme to the extent that the SB conformation, even in the absence of substrate, is preferred.

In light of the above observations, it is interesting to note that, in the substrate-bound wild-type P450 BM3 structures, the substrates are in close contact with the side chain of Ala264. This suggests that the mechanism by which substrate binding switches P450 BM3 to a different conformational state does not involve simply the expulsion of water molecules from the substrate-binding cavity, but in addition exploits the force exerted by the substrate on Ala264, a residue that acts as a sensitive trigger for the conformational conversion. In the A264E mutant, unfavorable steric interactions of the glutamate side chain induce the switch to the SB conformation, without necessity for the substrate interaction. An alternative explanation that could be put forward is that the P450 BM3 heme domain is in rapid conformational equilibrium between the SF and SB conformations, with the equilibrium being strongly favored toward the SF conformation for wild-type P450 BM3 in the absence of substrate. In this model, the substrate binds preferentially to the SB conformation, effectively shifting the equilibrium toward this form as the substrate concentration is increased. This model is also consistent with the behavior observed for the A264E heme domain. For several substrates tested, the apparent binding constants ($K_d$) determined are considerably lower than those for the wild-type heme domain, indicating much tighter binding (17). Given that the SB conformation is favored in the fatty acid-free form of the A264E heme domain, the "tight-binding" form of the protein is overrepresented in solution with respect to that seen for wild-type P450 BM3 (Fig. 3).

A substrate binding-induced spin-state shift has been observed in a large number of P450 enzymes studied to date (see Refs. 25 and 26). It is generally accepted that this behavior serves to avoid the potentially dangerous generation of active oxygen species that would occur through binding and subsequent reduction of molecular oxygen in the absence of substrate. Spin-state change induces a positive change in the heme iron reduction potential, favoring electron transfer from the redox partner (6, 7). The molecular mechanism underlying this change in the heme iron spin state has been proposed to be either a direct displacement of the sixth ligand water molecule (as in P450cam) or a more indirect displacement via substrate-induced changes in the protein structure (as in P450 BM3) (11). We have shown that the fatty acid-free A264E structure does show all the structural hallmarks of a substrate-bound P450 BM3 enzyme, but in the absence of substrate. An intriguing difference regarding A264E is the fact that, in contrast to the substrate-bound wild-type enzyme, A264E does not show any significant high-spin character in either the solution or crystalline state. A low-spin configuration would be expected for the glutamate-ligated species, but both spectroscopic and crystallographic studies show that there is a large population of non-glutamate-ligated protein in the substrate-free A264E heme domain and that this species does not lose the water ligand and convert to the high-spin form despite the change to the SB conformation. This suggests strongly that, even in wild-type P450 BM3, the spin-state shift is a direct (rather than an indirect) result of substrate binding. The high-resolution N-palmitoylglycine-bound wild-type structure has lead to the sug-
The proposed higher water affinity of site H over the heme iron ligation site (designated site L) and the fact that these sites are mutually exclusive (so that only a single water molecule can bind at either site L or H at any given time) led to the proposal that site H effectively pulls the water molecule away from site L, leading to the observed shift in the heme iron coordination state and hence conversion to the high-spin form [11]. The A264E heme domain structure shows all ligands to site H to be in identical positions compared with the N-palmitoylglycine-bound wild-type P450 BM3 structure (Fig. 4 upper). However, the water molecule still occupies site L (the heme iron) for molecule B, whereas in molecule A, Glu264 ligates the heme iron. In both cases, site H remains unoccupied due to steric hindrance with either the water (in molecule B) or the Glu264 side chain (in molecule A) at site L. It can therefore be concluded that site L (heme iron) remains the higher affinity site for water in the absence of substrate in the SB conformation.

As has been pointed out previously [11], there are no direct steric clashes between the bound fatty acid substrates of P450 BM3 and the water molecule at site L. The palmitoleic acid-bound structure of wild-type P450 BM3 indicates that secondary conformational changes in the protein/substrate must occur following reduction of the heme iron because the substrate is too distant from the heme iron for oxidative attack at catalytically relevant positions on the fatty acid chain [8]. NMR studies of the substrate-bound form of ferrous, fatty acid-bound wild-type P450 BM3 are consistent with a significant reorientation of the substrate in this enzyme form [27]. It is clear, however, that, upon binding of the fatty acid analog N-palmitoylglycine, the surroundings of site L become more hydrophobic, decreasing the water affinity and ultimately shifting the water molecule to predominantly occupy site H. Spectroscopic studies of wild-type P450 BM3 at catalytically relevant temperatures (by both electronic absorption and resonance Raman) have shown that, even in the presence of apparently saturating concentrations of substrate, an equilibrium exists between the high-spin five-coordinate and the low-spin six-coordinate states of the heme iron. Depending on the nature of the fatty acid substrate used, varying amounts of low-spin heme iron are detected, with shorter chain saturated fatty acids (e.g. lauric acid) being less effective than longer chain ones (e.g. palmitic acid) at effecting the shift in the spin-state equilibrium toward the high-spin state [28, 29]. This clearly indicates how, even in the presence of substrate, the possibility exists for water remaining bound at the heme iron (site L), and the water affinity of this site is strongly dependent on the nature of the substrate, although all drive the L-H equilibrium toward site H.

Crystal Structure of the Substrate-bound Form—In contrast to the requirement for screening novel conditions to obtain

**Fig. 4. Stereo view of the active site of A264E.** Upper, overlay of the active site of A264E (blue) and N-palmitoylglycine-bound wild-type P450 BM3 (green) (Protein Data Bank code 1JPZ). For clarity, the heme macrocycle is displayed only for the A264E mutant. The pattern of hydrogen bonding to site H is indicated by the dotted lines. The ligating water molecule occupying site L in the A264E mutant is colored red. Lower, active-site structure of the palmitoleic acid-bound form of the A264E heme domain. Residues are colored according to residue type; the substrate is depicted in purple.
suitable crystals of the substrate-free A264E heme domain, the palmitoleate-bound form of A264E was found to crystallize in the same space group as that reported previously for substrate-bound wild-type P450 BM3 (8). As observed for the wild-type enzyme, the resolution and quality of the data obtained for this particular crystal form are rather poor by comparison with those for the substrate-free enzyme. Nevertheless, electron density clearly indicates no major changes between the palmitoleate-bound A264E and palmitoleate-bound wild-type P450 BM3 structures. The single exception is the fact, that in all four molecules in the asymmetric unit of the A264E structure, the Glu264 side chain ligates the heme iron (Fig. 4, lower). This finding indicates that the presence of substrate induces movement of the glutamate onto the iron to replace water as the sixth heme ligand and is completely consistent with the spectroscopic studies reported in the accompanying article (17). Addition of long-chain fatty acids perturbs the UV-visible absorption spectrum of the A264E to a greater extent than those observed for wild-type P450 BM3 (17, 28). From the substrate-free A264E structure, it is clear that two conformations are possible for the Glu264 side chain, and solution studies indicate the equilibrium ratio between both states to be strongly dependent on the solution conditions. Upon palmitoleate binding, the substrate effectively occupies the volume of the non-heme iron-ligating conformation, driving the enzyme toward a completely ligated state, as observed in solution studies. Specifically, palmitoleate interacts with Phe87, preventing Glu264 from occupying the position observed in molecule A in the substrate-free A264E structure. Despite any significant further structural rearrangement induced following palmitoleic acid binding to the SB conformation of A264E, the substrate does influence directly the heme iron ligation state by minimizing the degrees of freedom available to the Glu264 side chain. Strong heme ligands such as azoles function as potent inhibitors for P450 enzymes, and many are used as antifungal drugs to inactivate the sterol demethylase P450 BM3 (30). It is conceivable that, despite the fact that the substrate-bound oxidized A264E structure shows fully six-coordinate heme iron, catalytic turnover for this mutant can still be observed, albeit at lower levels than those observed for wild-type P450 BM3 (17). This is a further indication that, upon reduction of ferric to ferrous iron, the position of the substrate with respect to the heme and perhaps the structure of the enzyme itself change dramatically, releasing the strong conformational lock on the glutamate side chain and allowing the substrate to bind to the iron, because this commits the enzyme to its "regular" catalytic cycle and prevents coordination of the glutamate to the iron until it returns to a ferric form following product formation. A further interesting aspect of this study arises from the structural change and its effect on the thermodynamic properties of the P450. In both the substrate-free and arachidionate-bound forms of the A264E mutant, the reduction potential of the heme iron is approximately ~315 mV (see accompanying article (17)). By contrast, the reduction potential of the conformationally different substrate-free form of P450 BM3 is ~427 mV, rising to ~289 mV upon binding arachidonate and an extensive shift in the spin-state equilibrium toward the high-spin form (31). In A264E, the heme iron remains predominantly low-spin in both the substrate-free and substrate-bound states. Thus, a possibility that arises is that the conformational change and its effects on the electronics of the heme system are of considerable importance in controlling the reduction potential of the heme iron. This is under further study using Ala264E variants in which the side chain of the introduced amino acid does not ligate the heme iron in the substrate-free and substrate-bound forms.

Further scrutiny of both the substrate-free and substrate-bound A264E structures reveals other important features of the P450 BM3 structure that relate to attempts to engineer covalent ligation of the heme macrocycle via the interaction of Glu264 with the heme 5-methyl group. The active-site organization in P450 BM3 is such that Phe87 and likely the I helix residue Thr260 obstruct access of Glu264 to the relevant position on the porphyrin ring. The failure to obtain any significant degree of covalent ligation in the A264E mutant may thus be explicable through steric restrictions in the active site. To A264E mutation, favors this conformational rearrangement induced by Glu264 and produce a more robust and biotechnologically exploitable form of P450 BM3, we are currently generating secondary mutations at these locations that might facilitate access of Glu264 to the relevant methyl group and could thus allow autocatalytic linkage to occur.

Conclusion—Crystallographic studies of the A264E variant of P450 BM3 confirm the proposals based on spectroscopic studies that the glutamate is able to ligate the ferric heme iron of the substrate-free form and that substrate addition "forces" on the ligand, producing a completely low-spin six-coordinate species, as opposed to the extensively high-spin five-coordinate form seen for the wild-type P450 BM3 (17). Structural studies explain clearly why substrate has this effect on the A264E enzyme since palmitoleic acid occupies one of the favored positions for the Glu264 side chain. Glu264 can no longer form an interaction with the key resiguene-specificity-determining residue Phe87 in the palmitoleate-bound form and is thus induced to move toward its only other acceptable position, coordinating to the heme iron.

An unexpected finding, but one with enormous ramifications for understanding the conformational changes that occur in P450 BM3 (and P450 systems in general) and their consequences, is the fact that both the substrate-free and palmitoleic acid-bound forms of the A264E heme domain have overall structural conformations that are virtually identical to those found for the substrate-bound forms of wild-type P450 BM3, but are distinct from that of the substrate-free wild-type heme domain (8, 11, 15). This SB conformation is not dependent on whether Glu264 ligates the heme iron or is positioned against Phe87, and the enzyme is low-spin in both forms and water-coordinated in the latter form for substrate-free A264E. The most obvious explanations are 1) that the SB conformation in wild-type P450 BM3 is a consequence of substrate-induced deformation of the I helix in the region of Ala264 and that the A264E mutation favors this conformational rearrangement independent of the substrate due to steric restrictions to movement of the glutamate side chain in the SB conformation, and/or 2) that P450 BM3 is in a continual dynamic equilibrium between the SF and SB conformations and that the A264E mutation forces this equilibrium toward the SB conformation. For both cases, the fact that the mutant remains in a low-spin form in the SB conformation in the fatty acid-bound structure suggests that the spin-state conversion observed in wild-type P450 BM3 upon substrate association (and the concomitant change in the reduction potential) is a consequence of the physical presence of the lipid in the environment of the heme, and not a result of the adoption of the SB conformation per se. Moreover, the fact that the SB conformation is clearly accessi-
able in the substrate-free A264E mutant also suggests that the binding of fatty acid might not be essential for inducing this conformational rearrangement in the wild-type enzyme and that the adoption of the SB conformation in the palmitoleate-bound wild-type structure could merely be a consequence of favorable binding of the substrate to this conformer. This conclusion is supported by the fact that much lower $K_d$ values are observed for binding of several long-chain fatty acids to the A264E variant than to the wild-type P450 BM3 (17). The SB conformation predominates in the mutant. In ongoing work, we aim to validate further the hypotheses that arise from these findings through creation of other variants at position 264, specifically investigating Ala$^{364}$ variants that induce the conformational switch to the SB conformation, but that do not, in addition, give rise to coordination to the heme iron.

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