FEBS Letters 396 (1996) 196–200 FEBS 17701

Inhibitor/fatty acid interactions with cytochrome P-450 BM3

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Received 5 July 1996; revised version received 23 September 1996

Abstract The interaction of fatty acid substrate (palmitate) and inhibitor (metyrapone: 2-methyl-1,2-di-3-pyridyl-1-propanone) with cytochrome P-450 BM3 was analysed by UV-visible and circular dichroism spectroscopy, and by surface-enhanced resonance Raman scattering (SERRS). While visible spectroscopy provides information on the relative affinities of these compounds, SERRS provides additional novel data indicating palmitate-induced structural changes in the haem environment. SERRS also demonstrates that binding of both palmitate and the large nitrogenous ligand metyrapone occurs simultaneously to P-450 BM3 – highlighting the usefulness of this technique in probing haemoprotein active sites.

Key words: Cytochrome P450 BM3; SERRS spectroscopy; Substrate interaction; Heme environment; CD

1. Introduction

Cytochrome P-450 BM3 (P-450 102) from *Bacillus megaterium* is an unusual member of the P-450 monooxygenase superfamily of haemoprotein enzymes [1,2]. It is soluble (like all bacterial P-450s), but differs from other bacterial P-450s in two major respects. Firstly, while all other characterised bacterial P-450s are reduced by electrons from NADH via two other enzymes (a flavoprotein dehydrogenase and an iron sulphur ferredoxin), P-450 BM3 receives electrons from NADPH via a single protein (an FAD- and FMN-containing NADPH cytochrome P-450 reductase similar to the membranous enzyme used by mammalian membrane-bound hepatic P-450 systems) [3]. Secondly, the BM3 P-450 (N-terminal) is fused to the reductase (C-terminal) in a single polypeptide [4]. This fusion protein arrangement is unlike other well characterised P-450s, but is similar to nitric oxide synthase [5]. The haem domain of P-450 BM3 shares greater primary structure homology with mammalian P-450 fatty acid hydroxylases than with any other bacterial enzyme and utilises a eukaryotic-like reductase system. Thus, P-450 BM3 currently represents the most appropriate model structure for eukaryotic microsomal P-450 systems.

The structure of the haem domain of P-450 BM3 has been solved [6]. It exhibits typical P-450 properties – possessing a hexacoordinated low-spin ferric haem, ligated on the proximal face by a cysteine (Cys_{400}) and on the distal face by a weakly bound water molecule. The haem is positioned at the base of a solvent exposed channel between two α-helices (helices I and L). Access to the haem distal face can only occur along this long hydrophobic channel. Differential electron density map calculations using arachidonate confirmed this channel as the substrate binding site [6].

The ability to purify fully active intact P-450 BM3 and its haem-containing domain opens the way for detailed analysis of their active sites. In this paper, we have utilised visible absorption and CD spectroscopy to analyse the interaction of fatty acid substrates and the P-450 inhibitor metyrapone with the haem domain of P-450 BM3. The vibrational technique of SERRS has also been used. This sensitive technique combines the molecular specificity of resonance Raman with an enhancement in scattering efficiency gained by coupling of the oscillating electric field of incident and scattered light with surface plasmons (waves of electrons) on roughened metal (e.g. gold, silver or copper) surfaces. Enhancement factors as large as 10^8 over normal Raman scattering have been observed from haem chromophores adsorbed on silver colloids or electrodes [7,8].

2. Materials and methods

2.1. E. coli strains, plasmid and bacteriophage vectors

E. coli strain XL-1 Blue (*sup*E44, *hsd*R17, *rec*A1, *gyr*A46, *thi*, *rel*A1, *lac*$^-$, F′ [*pro*AB$^+$, *lac*I^q, *lac*ZΔM15, Tn10(tet^r)]) [9] was used for plasmid propagation and the overexpression of the gene encoding cytochrome P-450 BM3 (*cyp*102) and the PCR-generated sub-genes encoding its constituent haem 'domains'. Construction of plasmids used for expression of P-450 BM3 and its constituent domains in *E. coli.* has been described previously [10]. Briefly, pBM23 consists of a 5 kb segment of *B. megaterium* chromosomal DNA (containing the *CYP*102 gene) cloned as an *Xba*I-*Eco*RI restriction fragment into vector pUC119 [11] and expression is from the *Bacillus* promoter. Plasmid pBM20 was used for the expression of the haem-containing domain of P-450 BM3 (residues 1–472) and consists of an approx. 1.5 kb PCR fragment of *CYP*102 cut with *Eco*RI and *Bam*HI (to generate cohesive ends as with pBM27) and cloned into pUC118 [12] under the *lac* promoter. Other bacterial strains and vectors used in these studies have been described previously [10].

2.2. Molecular biology techniques

DNA manipulations, bacterial transformations and other molecular techniques were performed using standard methods [9].

2.3. Expression and purification of intact cytochrome P-450 BM3 and its constituent haem domain

E. coli transformants carrying plasmids encoding wild-type P-450 BM3 and its constituent haem domain were grown overnight to high cell density in Terrific Broth plus antibiotic (ampicillin) [12]. IPTG inducer (final concentration 100 μg/ml) was added to facilitate expression from plasmids pBM20 (encoding the haem-containing domain). Intact P-450 BM3 was expressed from plasmid pBM23 under the control of its own promoter and without induction, as previously described [10].

Approx. 30 g of wet cell pellets were the starting points for polypeptide purification. Following cell rupture using a French press (950 lb/inch^2), polypeptides were purified to homogeneity by successive

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Abbreviations: P450, cytochrome P450-linked monooxygenase; MOPS, morpholinopropanesulphonate; CD, circular dichroism; SERRS, surface-enhanced resonance Raman spectroscopy; high-spin, 5-coordinate high-spin haem; low-spin, 6-coordinate low-spin haem

PII S0014-5793(96)01095-2

steps of ammonium sulphate precipitation (20–60% fraction retained), ion-exchange chromatography on DEAE-Sephacel and either affinity chromatography on 2′,5′-ADP-Sepharose (intact P-450 BM3) or by affinity for Bio-Gel HTP (DNA grade) hydroxyapatite (haem domain) as previously described [10]. Purification of the polypeptides by gel filtration using Sephacryl S-300 HR was used as a final purification step, when required.

2.4. Spectroscopy, protein and enzyme assays

Protein concentrations were determined using the method of Bradford [13] and by the BCA technique [14] with BSA as standard. Cytochrome P-450 concentrations were estimated by the method of Omura and Sato [15] using $\varepsilon = 91$ mM^{-1} cm^{-1} at 450 nm for the reduced plus CO adduct. NADPH-dependent fatty acid hydroxylation was measured at 30°C in 20 mM MOPS buffer (pH 7.4) containing 100 mM KCl, using $\varepsilon = 6.2$ mM^{-1} cm^{-1} at 340 nm. The assay system contained 0.25 mM sodium palmitate or sodium laurate and 0.2 mM NADPH. CD spectra were recorded on a Jasco J-600 spectropolarimeter. Far UV CD spectra were analysed for secondary structure by the CONTIN procedure [16].

For SERRS spectroscopy, colloidal silver was prepared according to a method modified from that originally reported by Lee and Meisel [17]. The procedure has been extensively described in a recent publication [18]. The approximately monodispersed suspension formed was stable for 6–8 weeks. Fresh samples of enzymes (P-450 BM3 holoenzyme or BM3 haem domain (10–40 μM)) were prepared in 100 μl of 100 mM sodium phosphate buffer (pH 7.5). 10–20 μl of the protein solution was then added to 290–280 μl of ice-cold 100 mM sodium phosphate buffer (pH 5.8) and the solution left on ice for 15 min. This solution was added to 2 ml of cooled Ag colloid and incubated on ice. 35 μl of 1% w/v L-ascorbic acid (Sigma, >99%) was added, initiating aggregation of the colloid. SERR spectra were recorded at 2–6°C, 30 min after initial aggregation.

For substrate-bound SERRS studies, 12 μl of a 75 mM methanolic solution of palmitate was added to P-450 BM3 haem domain in 300 μl of 100 mM sodium phosphate buffer (see above). For inhibitor-bound studies, 15 μl of 5 mM metyrapone was added to both substrate-free and palmitate-bound protein. In both cases, SERRS determinations were carried out as described above.

Spectra-Physics argon ion (2020/2045) continuous wave lasers provided 50 mW of 514.5 nm excitation. Data acquisition and analysis equipment have been described elsewhere [18]. A scanning rate of 1 cm^{-1} s^{-1} was employed for spectral acquisition.

2.5. Materials

Molecular biology reagents were purchased from Boehringer or United States Biochemicals. DEAE-Sephacel was purchased from Pharmacia-LKB. Silver nitrate (>99%) was obtained from Aldrich. All other reagents and enzymes were obtained from Sigma.

3. Results

3.1. Visible spectroscopic analysis of fatty acid interaction with P-450 BM3

The haem domain of cytochrome P-450 BM3 exhibits visible absorbance properties typical of other P-450s, with an intense Soret band located at 419 nm and the smaller α and β bands at approx. 570 and 535 nm, respectively. The haem iron is essentially all low-spin until addition of substrate. On binding of palmitate and other substrates, a type I absorbance shift occurs – with the Soret band moving to 390 nm. The amount of absorbance change induced by the addition of known quantities of fatty acids allows calculation of the association constants (K_s) for various substrates. Such analysis indicates K_s values of approx. 90 μM for lauric acid, 2 μM for palmitic acid and 6 μM for arachidonic acid with the haem domain.

Table 1
Selected vibrational bands of the P-450 BM3 haem domain in the presence and absence of palmitate

Vibrational mode	Frequency (cm^{-1})	
	Substrate-free P-450	Palmitate-bound P-450
ν4	1372	1372
ν29	–	1400
ν3	1493	1493
ν2	1572	1570
$\nu_{C=C}$	1628	1628

P-450 BM3 haem domain was at 1.6×10^{-7} M. Palmitate was added to a final concentration of 3×10^{-3} M. Experiments were performed as stated in Section 2.

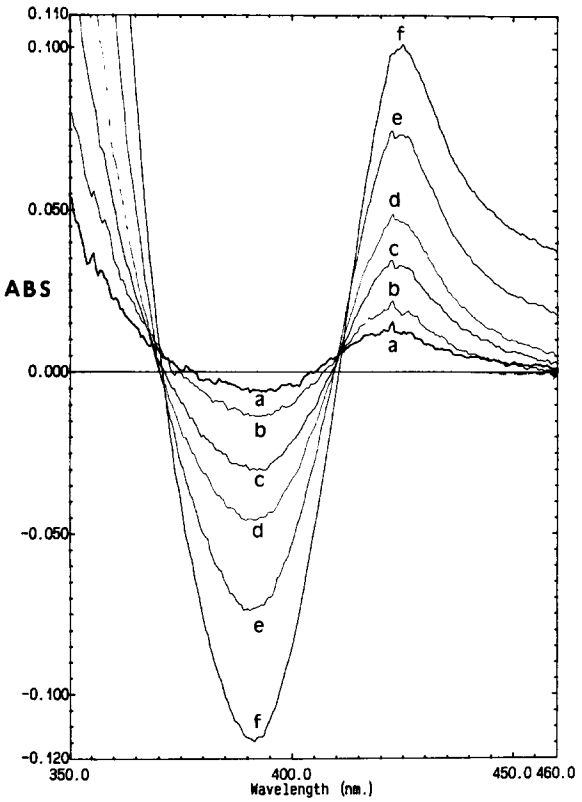

Fig. 1. Absorption difference spectra caused by titration of metyrapone into palmitate-bound P-450 BM3 haem domain. To cytochrome P-450 BM3 haem domain (0.8 μM) was added saturating (5 μM) palmitate in 20 mM MOPS (pH 7.4) containing 100 mM KCl at 30°C. Thereafter titration with metyrapone (from 100 mM aqueous stock solution) was performed – resulting in shift of the absorbance peak from 390 nm back to 419 nm. Spectra shown are at metyrapone concentrations of: (a) 0.6 mM, (b) 1.1 mM, (c) 2.04 mM, (d) 2.94 mM, (e) 5.06 mM and (f) 8.76 mM.

Addition of the P-450 inhibitor metyrapone to a solution of substrate-free P-450 BM3 haem domain does not result in a significant change in the visible absorption spectrum. However, titration of metyrapone this into a solution of fatty acid-bound enzyme induces high-spin to low-spin conversion in the haem iron, indicating the affinity of the inhibitor for the P-450 BM3 haem domain active site (Fig. 1). On the basis of inhibition of P-450 BM3 palmitate and laurate monooxygenase activities, the K_i for metyrapone was estimated at 4.1 ± 0.7 mM. Further, on the basis of the extent of high-spin to low-spin conversion of palmitate-bound P-450 BM3 haem domain caused by titration of metyrapone, the K_s for metyrapone was estimated at 9.8 ± 1.3 mM.

3.2. Circular dichroism studies on P-450 BM3 haem domain

CD spectra of cytochrome P-450 BM3 haem domain were recorded in the presence and absence of substrates. The substrate-free enzymes exhibit far UV CD patterns typical of proteins with a high α-helical content. Application of the CONTIN procedure [16] indicates that the protein consist of 34% α-helix. The addition of excess substrate (palmitate or arachidonate at 250 μM) does not perturb the far UV CD spectrum (approx. 190–260 nm) to any significant extent – indicating that addition of substrates does not result in major alterations in secondary structure, either through the binding of fatty acids in the active site or via interaction at the surface of the protein. It should also be noted that there is negligible change in the near UV CD signal between 260 and 320 nm on addition of fatty acids. This region of the spectrum provides information on the tertiary structure of proteins, and the lack of change indicates that no gross tertiary structural change occurs on fatty acid binding. In the visible CD range, the haem domain exhibits negative ellipticity over the majority of the spectrum from 300–500 nm. The spectrum of the haem domain has a large absorbance trough centred at 412 nm – a wavelength slightly different from that of the peak seen in the visible absorption spectrum (419 nm) (Fig. 2). Changes are clearly seen in the visible CD region after substrate addition. On addition of excess palmitic or arachidonic acid, there is a shift of the major absorption trough to lower wavelength (approx. 405 nm) (Fig. 2). Again, this wavelength is somewhat different from that at which the substrate-bound forms have absorbance maxima in the electronic absorption spectrum (390 nm). Despite its clear inhibitory action, the addition of metyrapone does not cause obvious changes in the far UV or visible CD spectra.

3.3. SERR spectroscopy on cytochrome P-450 BM3 haem domain

SERRS of substrate-free P-450 BM3 haem domain was obtained with excitation at 514.5 nm (Table 1). The vibrational nomenclature is adopted from Abe et al. [19]. The SERRS indicated a predominantly high-spin species for the protein adsorbed on the silver colloid. The position of spin-state sensitive bands v3 and v2 at 1493 and 1572 cm^{-1} confirms a contraction of the core ring indicative of a pentacoordinate porphyrin species [20]. Upon the addition of palmitate, further conversion to the high-spin state was observed. Additionally, a relative enhancement of the band at 1401 cm^{-1} occurred. This mode has been attributed to the v29 band of the porphyrin macrocycle (Table 1). The v29 vibration results from coordinate movements of peripheral methylene substituents, with iron-pyrollic nitrogen contributions. It is sensitive to structural alterations in the environment of the haem vinyl groups – and palmitate binding clearly causes such a structural perturbation.

Table 2
Vibrational bands for P-450 BM3 haem domain in the presence of palmitate and excess metyrapone (2.5×10^{-4} M)

Vibrational nomenclature[a]	Raman frequency (cm^{-1})
ν_a[b]	~1679
v10	1637; low-spin
v2	1580; low-spin
v3	1502; low-spin
v29	1400
v4	1372
ν_b[b]	1053
ν_c[b]	1032

Final P-450 and palmitate concentrations were 1.6×10^{-7} and 3×10^{-3} M, respectively. Experiments were performed as stated in Section 2.
[a]From [19].
[b]Vibrational mode from metyrapone.

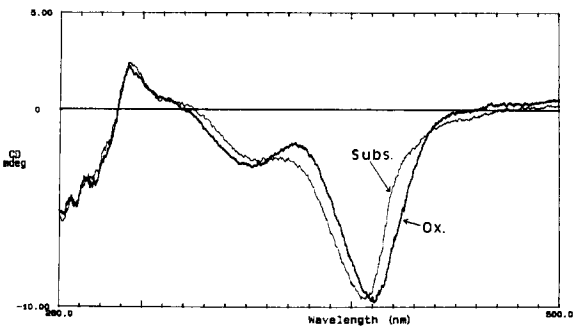

Fig. 2. Effect of palmitate addition on the visible region circular dichroism spectra of P-450 BM3 haem domain. The near UV and visible region CD spectrum of P-450 BM3 haem domain (1 μM) was recorded in 50 mM Tris (pH 7.5) at 25°C. Thereafter, palmitate was added to a final concentration of 200 μM. The substrate-free oxidised protein (Ox.) exhibits negative ellipticity over the entire Soret region of the CD spectrum (approx. 330 nm–460 nm). A shift in the position of the visible CD Soret band to lower wavelength occurs on addition of palmitate (Subs.) (CD minimum moving from 412 to 405 nm), reflecting the different haem environment in the substrate-bound haem domain.

Addition of an excess of the P-450 inhibitor metyrapone to P-450 BM3 haem domain causes a large conversion to the low-spin state, indicative of the ligation of the nitrogenous ligand, as demonstrated by the appearance of the v2, v3 and v10 spin-state marker bands at 1580, 1502 and 1637 cm^{-1}, respectively (Table 2). Vibrational bands assigned to metyrapone itself are positioned at 1679 cm^{-1} (ν_a), 1053 cm^{-1} (ν_b) and 1032 cm^{-1} (ν_c) (Fig. 3). Addition of metyrapone to palmitate-bound haem domain also results in the formation of a low-spin species. The presence of the enhanced v29 band at 1400 cm^{-1} (a fatty acid-induced vibration) indicates that the substrate is still retained within the pocket.

4. Discussion

Substrate-free P-450 BM3 haem domain adsorbed onto colloid yields SERR signals which are dominated by a high-spin species. SERRS signals are specific to the haem chromophore. Upon addition of metyrapone to palmitate-bound haem domain, conversion to the low-spin haem species occurs. The inference is that metyrapone has penetrated into the palmitate-containing pocket, combining with the iron and causing formation of the hexacoordinated low-spin ferric haem. The low-spin conversion supports metyrapone binding to haem iron [21,22] although no low frequency band has been assigned to metyrapone ligated to the haem. This is in agreement with the UV-visible and CD data, where the natural form of the protein observed in solution is low-spin and no significant changes are observed in the signals on addition of metyrapone.

The presence of palmitate within the pocket of this low-spin haem is demonstrated by the appearance of the mode assigned to v29 at 1400 cm^{-1}. The SERRS intensity of the v29 mode

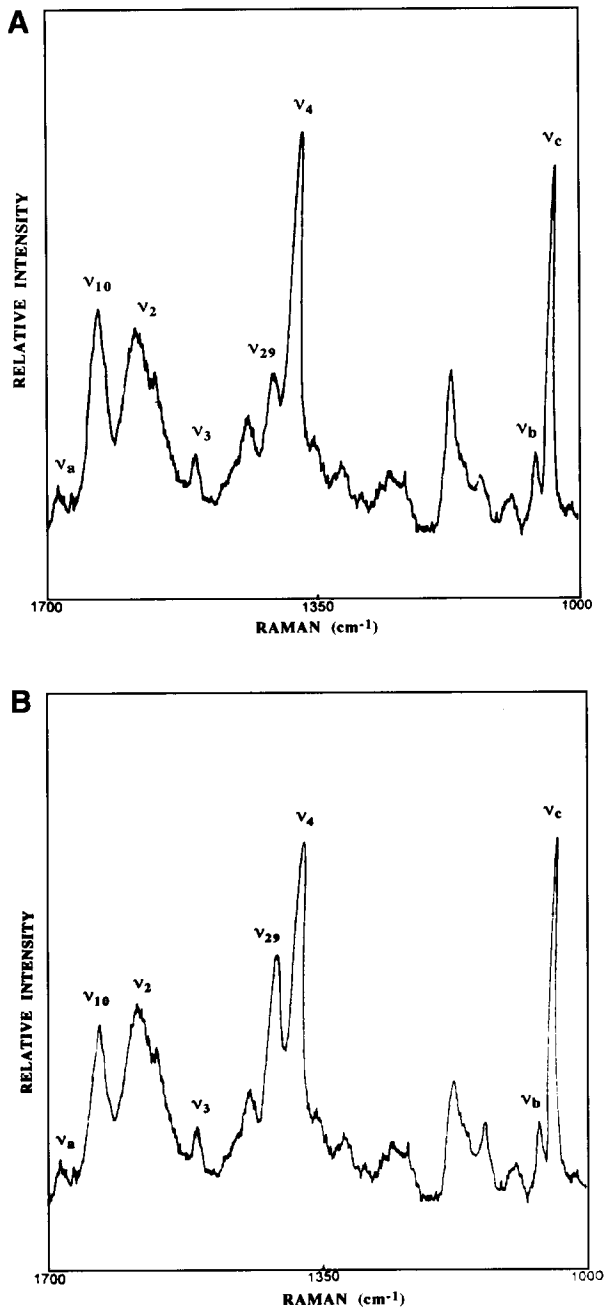

Fig. 3. SERRS spectra of metyrapone-bound and metyrapone/palmitate-bound P-450 BM3 haem domain. The concentration of protein used was 1.6×10^{-7} M. (a) Spectrum of the metyrapone-bound species (metyrapone concentration=3.3×10^{-5} M). (b) Spectrum resulting from addition of both metyrapone (3.3×10^{-5} M) and palmitate (5×10^{-4} M). Band assignment is identical to that in Table 2. Samples were prepared as described in Section 2.

relative to v4 increases upon the addition of palmitate to the P-450 BM3 haem domain. It is probable that the hydrophobic substrate positions itself against the rigid β-sheet of the binding pocket, held in place by non-ionic interactions which prevent haem/palmitate interaction. This binding would alter amino acid secondary and tertiary structural alignments. Allosteric transmission of this effect to the ferriprotoporphyrin IX ring, via amino acids in close proximity to the haem, may result in an enhancement of the sensitive v29 mode.

Visible absorption and visible CD studies show changes in the Soret region associated with the binding of fatty acids. However, far UV CD is not sufficiently sensitive to detect any haem pocket deformations associated with their binding. By contrast, SERRS indicates that binding of palmitate (and other fatty acids) to the haem domain causes enhancement of modes such as v29 (which is sensitive to the nature of the interaction of the haem ring with the immediate protein environment) but does not indicate direct interaction with the iron (no changes in v3 or v4). This finding is in agreement with the NMR data of Modi et al. [23] and suggests an allosteric mechanism for v29 intensity increase. It would appear that, despite its distance, the binding of fatty acid is 'sensed' by the haem group of the enzyme, most likely through small deformations in the amino acid environment of the fatty acid being transmitted through to the haem. On the basis of potentiometric studies performed with P-450cam, it is likely that fatty acid binding of induces an increase in the midpoint potential of the P-450 BM3 haem iron, such that it rises above that of reduced FMN in the reductase domain and can accept electrons from this flavin. The low-spin to high-spin conversion caused by fatty acid binding is thought to involve the removal of a water molecule from the coordination sphere of the haem iron.

Addition of metyrapone to cytochrome P-450 BM3 inhibits fatty acid hydroxylation. However, the binding of metyrapone does not cause obvious changes in the visible spectra of P-450 BM3. SERRS clearly demonstrates band changes associated with the binding of metyrapone alone (changes in v3 and v10) and palmitate alone (change in v29) to the active site of P-450 BM3 haem domain. Moreover, the fact that both sets of changes are observed in the presence of fatty acid and metyrapone indicates that the active site of P-450 BM3 is sufficiently large to accommodate both compounds at once.

The data presented demonstrate the usefulness of the spectroscopic technique of SERR in the analysis of molecular association with P-450 BM3. Fluorescence from the protein is quenched on the surface and only small quantities of enzyme (nM) are required. By contrast, resonance Raman spectroscopy demands micromolar amounts of enzyme. In addition, the technique has provided information on the binding of compounds which is not forthcoming from other spectroscopic techniques. Neither the binding of metyrapone nor the fatty acid-induced haem conformational change is detectable by UV-visible absorption spectroscopy or CD in any region of the spectrum. SERR spectroscopy identifies both phenomena, and provides evidence that simultaneous binding of fatty acid and metyrapone can occur in the P-450 BM3 active site.

Modi et al. [23], used NMR to show that lauric acid was bound at nearly 0.8 nm from iron in oxidised P-450 BM3, sufficiently far to permit the co-binding of pyridine. This situation is different from P-450cam, where camphor/pyridine co-binding is not feasible. Only small ligands (such as cyanide) can bind simultaneously with camphor [24]. In this manuscript, we have used SERR spectroscopy to demonstrate that co-binding of the larger inhibitor molecule metyrapone is also feasible in the presence of palmitate. Fatty acid substrates in P-450 BM3 are bound at too great a distance from the haem for monooxygenation to occur. It appears that reduction of fatty acid-bound P-450 BM3 brings about a movement of substrate closer to the haem and facilitates oxygenation at the ω-1, ω-2 or ω-3 positions [25]. This process may

involve the movement of residue F87, a putative electron transfer pathway amino acid, which protrudes into the active site of P-450 BM3 above the haem iron [6]. We are currently addressing this problem with SERR and other spectroscopic analyses of P-450 BM3 mutants, including those at residue F87.

Acknowledgements: We wish to thank the BBSRC and the Wellcome Trust (studentship to I.D.G.M.) for financial support. A.W.M. holds a Royal Society of Edinburgh Caledonian Research Fellowship. The assistance of Professor N.C. Price and Dr. S.M. Kelly at the BBSRC CD facility (University of Stirling) is also greatly appreciated. Finally, the authors wish to acknowledge Professor J.R. Coggins and Professor J.G. Lindsay for support in our research.

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