Effect of replacement of ferriprotoporphyrin IX in the haem domain of cytochrome *P*-450 BM-3 on substrate binding and catalytic activity

Sandeep MODI,*† William U. PRIMROSE,* Lu-Yun LIAN* and Gordon C. K. Roberts*†

*Department of Biochemistry and Biological NMR Centre and †Centre for Mechanisms of Human Toxicity, University of Leicester, Leicester LE1 9HN, U.K.

Bacillus megaterium cytochrome *P*-450 BM-3 (coded by gene *CYP102*) is a catalytically self-sufficient mono-oxygenase, with both cytochrome *P*-450 and NADPH:cytochrome *P*-450 reductase domains, that catalyses the hydroxylation of fatty acids. The natural ferriprotoporphyrin IX has been removed from the haem domain of cytochrome *P*-450 BM-3 by treatment with acidified acetone, and it has been shown that, under carefully controlled conditions, haem can be added back to the resultant apoprotein to obtain a fully reconstituted haem domain with spectroscopic, substrate-binding and catalytic properties indis-

tinguishable from those of the native domain. Replacement of the natural haem with ferriprotoporphyrin IX dimethyl ester yields a protein which has a higher affinity for the substrate dodecanoic acid and (in the presence of the reductase domain) the same catalytic rate as the native haem domain. Replacement with ferrimesoporphyrin IX yields a protein with the same affinity for substrate, but a reduced catalytic turnover. These results suggest that the haem moiety has a role in the creation of the binding pocket for substrate, and that modification of the electron density on the haem iron effects the catalytic rate.

INTRODUCTION

Cytochrome *P*-450 (*P*-450) BM-3 (EC 1.14.14.1) from *Bacillus megaterium* is a unique bacterial model for the *P*-450s of the mammalian endoplasmic reticulum which are involved in drug and xenobiotic metabolism. It is a catalytically self-sufficient mono-oxygenase which catalyses hydroxylation at the $\omega-1$, $\omega-2$ and $\omega-3$ positions of long-chain saturated and mono-unsaturated fatty acids, alcohols and amides, and the epoxidation and/or hydroxylation of medium-chain monounsaturated fatty acids [1–4]. The enzyme can be cleaved by trypsin into two distinct domains: an N-terminal ‘haem domain’ (M_r 54000), containing haem (ferriprotoporphyrin IX), and a C-terminal flavoprotein ‘reductase domain’ (M_r 64000), which contains FAD, FMN and the NADPH-binding site [5]. The gene coding for *B. megaterium* *P*-450 BM-3 (*CYP102*) has been cloned and sequenced [6–7] and expression systems for the individual domains have been developed [5,8,9]. The separate domains have been found to be functionally active; the haem domain is capable of binding substrates, while the reductase domain is capable of reducing cytochrome *c* [9]. The mono-oxygenase activity characteristic of the intact *P*-450 BM-3 can be reconstituted using the individual recombinant domains, although the activity of the reconstituted system reported by different authors varies markedly: Munro et al. [10] reported an activity, measured by NADPH consumption, corresponding to 0.1–0.3 % of that of the intact enzyme activity, whereas Boddupalli et al. [11] reported an activity corresponding to 80 % of that of the intact enzyme, as measured by oxygen consumption. Both domains show clear sequence similarity to the corresponding mammalian microsomal enzymes [7].

Notwithstanding the central importance of the haem of *P*-450s to their function, little is yet known of the structure–function relationships of the haem in these enzymes, since it has proved very difficult to remove and replace the natural ferriprotoporphyrin IX by other haems. For example, in *P*-450 cam the haem could be removed to yield the apoprotein, but after the haem was replaced, only 66 % of the enzyme was catalytically active, owing in part to conversion into the inactive *P*-420 form

[12]. In the present paper we report a method for the removal and replacement of ferriprotoporphyrin IX from the haem domain of *P*-450 BM-3. For the first time we show that the resulting apoprotein can be fully reconstituted into active *P*-450 by addition of haem under appropriate conditions. We also report the replacement of protoporphyrin IX by two other haems (mesoporphyrin and protoporphyrin dimethyl ester) and the study of the effects of this on substrate binding and catalytic activity.

EXPERIMENTAL

Materials

Hydroxyapatite resin was purchased from Bio-Rad, isopropyl β -D-thiogalactopyranoside from NovoChem, and DEAE-Sephacel, PD10 columns and Sephacryl S-300 from Pharmacia LKB. Ferriprotoporphyrin IX, ferrimesoporphyrin IX and ferriprotoporphyrin IX dimethyl ester, dodecanoic (lauric) acid sodium salt and NADPH were purchased from Sigma Chemical Co. All other chemicals used were purchased from either Fisons or Sigma and were at least of analytical grade.

Enzyme production and purification

Plasmids pJM23 (encoding intact *P*-450 BM-3), pJM20 (encoding the *P*-450 domain of *P*-450 BM-3), and pJM27 (encoding the reductase domain of *P*-450 BM-3) were kindly provided by Dr. J. S. Miles, Department of Biochemistry, University of Glasgow, Glasgow, Scotland, U.K. The intact *P*-450 BM-3 and its haem and reductase domains were expressed from these vectors in *Escherichia coli* XL1- Blue, genotype *supE44*, *hsdR17*, *recA1*, *endA1*, *gyrA46*, *thi*, *relA1*, *lac*[−] F' [*proAB*⁺ *lac*[−] *lacZ*DM15 *Tn10* (*tet*^r)]. Proteins were purified to homogeneity essentially as previously described [9,13]. Protein purity was confirmed by SDS/PAGE, staining with Coomassie Blue, and by the ratio A_{418}/A_{280} , where a high level of purity is represented by a ratio of 0.7 or above for the intact *P*-450 BM-3, and 1.7 or above for

its *P*-450 domain (A. W. Munro, personal communication). Protein concentrations were measured by the method of Omura and Sato [14] using $\epsilon = 96 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 419 nm for intact *P*-450 BM-3, $\epsilon = 77.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 418 nm for the haem domain, and $\epsilon = 21.2 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 456 nm for the reductase domain [15].

Enzyme assay

NADPH-dependent fatty acid hydroxylation catalysed by cytochrome *P*-450 BM-3 was measured spectrophotometrically as NADPH oxidation as described by Matson et al. [16]. The assay mixture contained 20–1000 μM sodium dodecanoate and 200 μM NADPH in 0.1 M phosphate buffer (pH 6.5–9.0) at 23 °C. The activity was determined from the decrease in absorbance at 340 nm using $\Delta\epsilon = 6.2 \text{ mM}^{-1} \cdot \text{cm}^{-1}$. The activity of intact cytochrome *P*-450 BM-3 towards sodium dodecanoate was measured as a function of pH; the k_{cat} at pH 8.0 was found to be $1540 (\pm 120) \text{ min}^{-1}$, in good agreement with earlier reports [5,9] and to be maximum over the pH range 7.5–8.5. Therefore, all subsequent experiments were performed at pH 8.0. The catalytic activity of the separated haem and reductase domains was determined by mixing the domains in 0.1 M phosphate buffer, pH 8.0, containing 20 mM KCl.

Removal and replacement of haem

A 100 mg portion of the haem domain of cytochrome *P*-450 BM-3 in 5 ml of 50 mM phosphate buffer, pH 7.0, was added dropwise with constant stirring to a cooled (-20°C) solution of 50 ml of acetone acidified with 0.1 ml of conc. HCl. After 10 min the solution was centrifuged at 10000 *g* for 5 min at -20°C . The precipitate was dissolved in 2 ml of deoxygenated 0.06 M KOH solution containing 2 mM dithiothreitol (DTT) and then dialysed against 2 litres of the same solution at 4 °C under nitrogen for 5 h. The resultant apoprotein was further dialysed against 2 litres of a deoxygenated solution of 4 M guanidinium hydrochloride at 4 °C.

To regenerate the holoprotein, a 1.1-fold molar excess of ferriprotoporphyrin IX, ferrimesoporphyrin or ferriprotoporphyrin dimethyl ester in 100 μl of dimethylformamide was added to 4 ml of a 25 mg/ml solution of the apoprotein in 2 mM DTT/0.1 M phosphate, pH 7.0, under nitrogen and left at 4 °C for 16 h with constant stirring. The resulting solution was dialysed against 2 litres of deoxygenated 0.1 M phosphate buffer, pH 7.0, containing 2 mM DTT and a 1.1-fold excess of the appropriate haem, at 4 °C under nitrogen. Excess of unbound haem was then removed by ion-exchange chromatography on a DEAE-Sephacel column, elution being performed with a gradient of 0–0.5 M KCl in 0.1 M phosphate buffer, pH 7.0, containing 2 mM DTT. Finally, the protein solution was dialysed at 4 °C against 40% glycerol/0.1 M phosphate buffer, pH 7.0, containing 100 mg/ml cysteine, to convert any remaining *P*-420 into *P*-450 [12]. The haem domain of cytochrome *P*-450 BM-3 reconstituted so as to contain ferrimesoporphyrin or ferriprotoporphyrin dimethyl ester was found to be stable under these conditions at 4 °C for at least 24 h; for long-term storage at -20°C it was transferred to 50% glycerol/0.1 M phosphate buffer, pH 7.0, containing 2 mM DTT. Concentrations of the reconstituted domains were measured by the method of Omura and Sato [14] using $\epsilon = 91 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 448 nm for the CO complex of the ferrimesoporphyrin-containing domain and at 450 nm for the CO complex of the ferriprotoporphyrin dimethyl ester-containing domain.

Optical spectroscopy

Since the optimum pH for the hydroxylation of dodecanoic acid by cytochrome *P*-450 BM-3 is between pH 7.0 and 9.0, optical spectroscopy was carried out at pH 8.0. Spectra were obtained using a Beckman DU 650 spectrophotometer with quartz cells (10 mm pathlength). Titrations were carried out at 23 °C by the addition of substrate (final concn. 20 μM –1.5 mM) to the enzyme (10–15 μM) in 0.1 M phosphate buffer, pH 8.0, containing 20 mM KCl, in the sample cell, while adding the same volume of buffer alone to the same concentration of enzyme in the reference cell. Dissociation equilibrium constants were estimated using the following equation [17–19]:

$$1/\Delta A = (K_d/\Delta A_\infty)(1/S_0) + (1/\Delta A_\infty) \quad (1)$$

where ΔA and ΔA_∞ are the changes in absorbance at the observation wavelength (418 nm) at the given (S_0) and saturating substrate concentrations respectively, and K_d is the dissociation constant of the enzyme–substrate complex. K_d and ΔA_∞ were evaluated from the slope and intercept of a plot of $1/\Delta A$ versus $1/S_0$.

RESULTS AND DISCUSSION

Haem was removed from the haem domain of cytochrome *P*-450 BM-3 by treatment with acidified acetone followed by dialysis, as described in the Experimental section. The optical spectrum of the apoprotein obtained from the haem domain is shown in Figure 1. The absence of any absorbance in the visible region of the spectrum of the apo domain indicates the complete removal of haem. Regeneration of the holoprotein by adding back ferriprotoporphyrin IX to the apo domain under the conditions described in the Experimental section resulted in an optical spectrum (Figure 1) identical with that of the untreated haem domain of cytochrome *P*-450 BM-3 (in Figure 1 the two traces overlap perfectly, so only one trace is seen). On reduction of the regenerated haem domain with sodium dithionite and addition of CO, the Soret band was found at 450 nm, and there was no evidence for the formation of *P*-420, the inactive form of cytochrome *P*-450 with a characteristic absorption maximum at 420 nm (Figure 1). The reconstituted haem domain was found to

Figure 1 UV-visible absorption spectra of the haem domain of cytochrome *P*-450 BM-3

-----, Haem domain after removal of haem ([protein] = 11.3 μM); —, untreated haem domain and haem domain reconstituted with ferriprotoporphyrin IX ([protein] = 5.93 μM); reduced CO complex of reconstituted haem domain.

Table 1 Dodecanoic acid binding and hydroxylation by native and reconstituted haem domain of cytochrome *P*-450 BM-3

K_m and k_{cat} values were measured by NADPH oxidation, and K_d values by optical spectroscopy, in 0.1M phosphate buffer (pH 8.0)/20 mM KCl at 23 °C, except for the intact enzyme, for which the KCl was omitted. The ratio of reductase to haem domain was 1:12.

Enzyme	K_m (μ M)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{M}^{-1} \cdot \text{s}^{-1}$)	K_d (μ M)
Intact cytochrome <i>P</i> -450 BM-3	130 $\pm$ 10	1540 $\pm$ 120	2×10^5	290 $\pm$ 25
Haem domain + reductase domain				
Untreated haem domain	924 $\pm$ 87	4.5 $\pm$ 0.4	81	1026 $\pm$ 93
Reconstituted with haem	963 $\pm$ 82	4.2 $\pm$ 0.3	73	964 $\pm$ 80
Reconstituted with mesohaem	799 $\pm$ 95	2.6 $\pm$ 0.3	54	959 $\pm$ 86
Reconstituted with haem dimethyl ester	258 $\pm$ 26	4.2 $\pm$ 0.4	271	241 $\pm$ 21

Table 2 Optical absorption maxima of intact and of native and reconstituted haem domains of cytochrome *P*-450 BM-3

Enzyme	Absorption maxima (nm)	
	Oxidized enzyme	Reduced CO complex
Intact cytochrome <i>P</i> -450 BM-3	419, 534, 568	449, 549
Native haem domain	418, 535, 568	449, 549
Mesohaem haem domain	413, 534, 567	448, 549
Haem dimethyl ester haem domain	418, 535, 568	450, 549

have essentially the same dissociation constant for substrate binding to the ferric form as the untreated domain (Table 1).

The catalytic activity of the reconstituted haem domain was measured by the rate of substrate-dependent NADPH oxidation in the presence of the reductase domain of cytochrome *P*-450 BM-3. In our hands, the maximum catalytic activity observed when mixing the two (untreated) domains of cytochrome *P*-450 BM-3 in ratios of either 60 μ M reductase to 5 μ M haem domain or 60 μ M haem domain to 5 μ M reductase was 4.5–4.9 nmol/min per nmol of haem. This is about 300 times less than that observed for intact cytochrome *P*-450 BM-3, in agreement with the results of Munro et al. [10]. At a concentration of 5 μ M of each domain, k_{cat} was 1.3 nmol/min per nmol of haem; activity increased as the concentration of either component was increased (keeping the other at 5 μ M), to the maximum values given above at ratios of 12:1. A further increase to 20:1 did not significantly increase the k_{cat} , suggesting that the low activity of the separated domains may not be due solely to their low affinity for one another; it is possible that in the intact enzyme the linker sequence contributes to the correct orientation of the domains. The values given for substrate-dependent NADPH oxidation by the mixed domains were obtained at 100 mM phosphate/20 mM KCl; this gave better results than 100 mM phosphate alone, but further addition of KCl decreased the observed rate. As shown in Table 1, the activity of the reconstituted haem domain in this assay was indistinguishable from that of the untreated domain.

This is the first report of removal and replacement of the haem moiety resulting in a fully active cytochrome *P*-450. Earlier attempts, by treating cytochrome *P*-450 solutions with apomyoglobin [20] or with acidified acetone [12], led to no more than 66% recovery of active cytochrome *P*-450. We find that the presence of DTT during reconstitution of the apo domain is essential; its omission leads to formation of the inactive *P*-420

Figure 2 UV-visible absorption spectra of the intact haem domain of cytochrome *P*-450 BM-3 (16.13 μ M, —) and the haem domain reconstituted with mesohaem (16.13 μ M, ----)

species. The method described here was not successful with the more complex intact cytochrome *P*-450 BM-3, owing to instability of the protein and the loss of the FAD and FMN cofactors from the reductase domain (results not shown).

The apoprotein obtained from the haem domain was reconstituted in a similar manner with two other haems, ferri-mesoporphyrin (mesohaem) and ferriprotoporphyrin IX dimethyl ester (haem dimethyl ester). Table 2 lists the wavelengths of absorption maxima for the haem domain of cytochrome *P*-450 BM-3 reconstituted with different haems, both in their oxidized forms and in their reduced complexes with CO. The optical spectra of the domain containing haem dimethyl ester were found to be very similar to those of the native haem domain, while those of the domain containing mesohaem were slightly different (Figure 2), in line with the difference in the spectra of the isolated porphyrins (results not shown).

Figure 3 shows the electronic absorption spectra of the haem domain containing haem dimethyl ester in the absence or presence of dodecanoic acid. Addition of substrate causes the Soret band at 418 nm to decrease, while the absorption increases at 394 nm and at 650 nm. The band at 650 nm is characteristic of high-spin ferric haem proteins [21]. The binding of dodecanoic acid is accompanied by the expulsion of a water molecule from the sixth co-ordination position of the haem iron [13], and the ferric iron

Figure 3 Effect of dodecanoate on the UV-visible absorption spectra of the haem domain of cytochrome *P*-450 BM-3 reconstituted with haem dimethyl ester

(a) Spectra of the haem domain containing haem dimethyl ester (14.3 μ M) in the absence (—) or presence of sodium dodecanoate (1.2 mM, ----). (b) Change in absorbance (ΔA) at 418 nm as a function of dodecanoate concentration (S_0). The line is the least-squares fit of eqn. (1) to the experimental data.

goes from low-spin ($S = 1/2$) to high-spin ($S = 5/2$) [9]. It is clear from the spectra in Figure 3 that these changes also occur when dodecanoic acid binds to the haem domain containing haem dimethyl ester.

The functional properties of the haem domain of cytochrome *P*-450 BM-3 reconstituted so as to contain either normal haem, mesohaem or haem dimethyl ester, in terms of their affinity for dodecanoic acid and their catalytic activity (in the presence of the reductase domain) are summarized in Table 1. Although, as noted above, the substrate-dependent NADPH oxidation by the reconstituted domains is very low compared with that of the intact enzyme, they are still sufficiently active to enable comparisons to be made between haem domains regenerated with different haems. The equilibrium dissociation constants for dodecanoate binding (K_d) were determined from the changes in absorption spectra on substrate binding (Figure 3). The affinity of the isolated haem domain for the substrate is slightly (≈ 3.5 -fold) lower than that of the intact enzyme.

The haem domain containing haem dimethyl ester has essentially the same catalytic activity as the native haem domain, but it binds substrate significantly (about 4-fold) more tightly. As a result, this substituted haem domain is, in terms of k_{cat}/K_m , a more effective catalyst than the native domain. In the X-ray crystal structure of the haem domain of cytochrome *P*-450 BM-3 [21] it can be seen that one of the propionic acid substituents of the haem is near the hydrophobic substrate-binding channel. We have recently used paramagnetic relaxation experiments in conjunction with the crystal structure to construct an approximate model for the binding of dodecanoate to the oxidized enzyme [13], and this is shown in Figure 4. In this model, one of the propionate side chains is seen to be close to the hydrocarbon chain of the bound substrate. Esterification of the haem propionate groups removes this negative charge from the vicinity of the fatty-acid-binding site, increasing its non-polar nature; this may explain the observed increase in the affinity for the substrate when haem dimethyl ester is substituted for haem in this domain. This in turn suggests that the substrate specificity of cytochrome *P*-450 BM-3 is determined, to a modest extent, by the haem as well as by the protein.

Figure 4 The substrate binding pocket of the haem domain of cytochrome *P*-450 BM-3, showing the proximity of one of the propionate substituents of the haem to the substrate bound to the enzyme

The haem is at the right; the amino acid residues which make up the substrate-binding pocket are shown in a stick representation, with dots representing the van der Waals' surface. The substrate, dodecanoic acid is shown in bold. Based on Ravichandran et al. [22] and Modi et al. [13].

When the haem is replaced by mesohaem, the binding of the substrate is not affected (although K_m is slightly lower; the difference between the K_a and K_m values, particularly in the intact enzyme, arises from the fact that they are likely to reflect substrate binding to different enzyme species), but the rate of NADPH oxidation in the presence of the reductase domain is about a factor of two slower than that seen with the native haem domain (Table 1). This suggests that the basicity, and hence the redox potential, of the haem plays a role in determining the rate of hydroxylation of substrate. Replacement of the vinyl groups of the protoporphyrin IX of native *P*-450 by ethyl groups in mesoporphyrin would be expected to lead to an increase in the electron density on the haem iron, and hence to a decrease in the rate of haem reduction. That this is directly reflected in a change in the catalytic rate suggests that haem reduction may be the rate-limiting step in substrate hydroxylation by the separated domains of cytochrome *P*-450 BM-3. It remains to be established whether a decreased rate of haem reduction by the reductase domain when the two domains are no longer covalently linked is the origin of the greatly decreased catalytic turnover of the separated domains as compared with the intact enzyme.

The availability of an efficient method for the removal and replacement of the haem in the haem domain of cytochrome *P*-450 BM-3 opens the way to detailed studies of the effects of haem structure on the function of this enzyme. The effects observed in the initial work reported here suggest that these may be expected to throw further light on the mechanisms of this important class of enzymes.

This work was supported by a grant from the Medical Research Council.

REFERENCES

- 1 Miura, Y. and Fulco, A. J. (1975) *Biochim. Biophys. Acta* **388**, 305–317
- 2 Ho, P. P. and Fulco, A. J. (1976) *Biochim. Biophys. Acta* **431**, 249–256
- 3 Narhi, L. O. and Fulco, A. J. (1986) *J. Biol. Chem.* **261**, 7160–7169
- 4 Fulco, A. J. (1991) *Annu. Rev. Pharmacol. Toxicol.* **31**, 177–203
- 5 Li, H., Darwish, K. and Poulos, T. L. (1991) *J. Biol. Chem.* **266**, 11909–11914
- 6 Wen, L.-P. and Fulco, A. J. (1987) *J. Biol. Chem.* **262**, 6676–6682
- 7 Ruettinger, R. T., Wen, L.-P. and Fulco, A. J. (1989) *J. Biol. Chem.* **264**, 10987–10995
- 8 Oster, T., Boddupalli, S. S. and Peterson, J. A. (1991) *J. Biol. Chem.* **266**, 22718–22725
- 9 Miles, J. S., Munro, A. W., Rosendowski, B. N., Smith, W. E., McKnight, J. and Thompson, A. J. (1992) *Biochem. J.* **288**, 503–509
- 10 Munro, A. W., Lindsay, J. G., Coggins, J. R., Kelly, S. M. and Price, N. C. (1994) *FEBS Lett.* **343**, 70–74
- 11 Boddupalli, S. S., Oster, T., Estabrook, R. W. and Peterson, J. A. (1992) *J. Biol. Chem.* **267**, 10375–10380
- 12 Yu, C. A. and Gunsalus, I. C. (1974) *J. Biol. Chem.* **249**, 107–110
- 13 Modi, S., Primrose, W. U., Boyle, J. M. B., Gibson, C. F., Lian L.-Y., and Roberts, G. C. K. (1995) *Biochemistry*, in the press
- 14 Omura, T., and Sato, R. (1964) *J. Biol. Chem.* **239**, 2379–2387
- 15 Vermilion, J. L. and Coon, M. J. (1978) *J. Biol. Chem.* **253**, 8812–8819
- 16 Matson, R. S., Hare, R. S. and Fulco, A. J. (1977) *Biochim. Biophys. Acta* **487**, 487–494
- 17 Sakurada, J., Takahashi, S., and Hosoya, T. (1986) *J. Biol. Chem.* **261**, 9657–9662
- 18 Modi, S., Behere, D. V. and Mitra, S. (1989) *Biochim. Biophys. Acta* **996**, 214–225
- 19 Saxena, A., Modi, S., Behere, D. V. and Mitra, S. (1990) *Biochim. Biophys. Acta* **1041**, 83–93
- 20 Müller-Eberhard, U., Liem, H. H., Yu, C. A. and Gunsalus, I. C. (1969) *Biochem. Biophys. Res. Commun.* **35**, 229–231
- 21 Falk, J. E. (1975) in *Porphyrins and Metalloporphyrins* (Smith, K. M., ed.), pp. 131–190, Elsevier, Amsterdam
- 22 Ravichandran, K. G., Boddupalli, S. S., Hasemann, C. A., Peterson, J. A. and Deisenhofer, J. (1993) *Science* **261**, 731–735