Biochem. J. (1992) 288, 503–509 (Printed in Great Britain)

Domains of the catalytically self-sufficient cytochrome *P*-450 BM-3

Genetic construction, overexpression, purification and spectroscopic characterization

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1. The gene *CYP102* encoding cytochrome *P*-450 BM-3 and subgenes encoding the cytochrome *P*-450 and cytochrome *P*-450 reductase domains have been cloned in *Escherichia coli*. 2. The protein products of these genes have been overexpressed and purified to homogeneity. 3. The cytochrome *P*-450 domain is purified in the ferric low-spin state, but is readily converted into the high-spin state by addition of the substrate palmitate (K_s = 1 μM). The cytochrome *P*-450 reductase domain readily reduces cytochrome *c*. Mixing the two domains reconstitutes only about one-thousandth of the fatty acid hydroxylase activity associated with the intact cytochrome *P*-450 BM-3. 4. The X-band e.p.r. spectra of both the cytochrome *P*-450 domain and intact cytochrome *P*-450 BM-3 give *g*-values indicating low-spin ferric haem. The spectra are virtually identical with those of the equivalent form of cytochrome *P*-450 cam indicating that the haem ligation in cytochrome *P*-450 BM-3 is identical with that of cytochrome *P*-450 cam. 5. Resonance Raman spectra of the substrate-free and substrate-bound forms of the cytochrome *P*-450 domain are given. Spectral differences in comparison with cytochrome *P*-450 cam may reflect subtle electronic differences between the respective haem environments.

INTRODUCTION

Cytochrome *P*-450-dependent mixed-function mono-oxygenases (*P*-450s) are a supergene family of enzymes that catalyse the oxidation of an extremely wide range of lipophilic chemicals by the addition of one atom of molecular oxygen into the substrate. They are widespread in nature and play a central role in the metabolism of endogenous compounds, such as steroids and fatty acids, and also a variety of foreign compounds [for reviews see Ortiz de Montellano (1986), Gonzalez (1989, 1990) and Nebert *et al.* (1991)]. In general, *P*-450s require a separate reductase system in order to function catalytically. For example, the microsomal *P*-450s require the flavoprotein *P*-450 reductase as an electron donor, whereas mitochondrial and most bacterial *P*-450s require an iron–sulphur-containing redoxin and an FAD-containing redoxin reductase. A so-far unique exception to this generalization is the 118 kDa fatty acid mono-oxygenating *P*-450 BM-3 from *Bacillus megaterium* ATCC 14581 (Narhi & Fulco, 1986).

P-450 BM-3 is a bifunctional polypeptide comprising a 54 kDa *N*-terminal '*P*-450' domain linked to a 64 kDa *C*-terminal 'reductase' domain and is therefore catalytically self-sufficient (Narhi & Fulco, 1986). The gene encoding *P*-450 BM-3 has been cloned and sequenced (*CYP102*; Wen & Fulco, 1987; Ruettinger *et al.*, 1989), and detailed comparisons of the predicted amino acid sequence have shown that the *P*-450 domain is most closely related to mammalian fatty acid-metabolizing *P*-450s of family 4 (25% identity) rather than any other bacterial *P*-450s (Nebert *et al.*, 1991). Similarly, the reductase domain is approx. 33% identical with *P*-450 reductases from several eukaryotic sources, including man, and contains all of the conserved residues associated with binding of the FAD and FMN cofactors, and NADPH (Ruettinger *et al.*, 1989). Clearly, this unique configuration suggests that *P*-450 BM-3 is a very attractive model for understanding many aspects of *P*-450 function, not least because it is soluble, unlike almost all eukaryotic *P*-450s, and it can be expressed to a very high level in *Escherichia coli* (Narhi *et al.*, 1988; Boddupalli *et al.*, 1990).

A further intriguing aspect of *P*-450 BM-3 is that it gives rise to two distinct hydroxylated products with lauric acid and myristic acid as substrate, but also catalyses multiple rounds of oxidation of palmitic acid, where the products of one round are the substrates for the next and the fatty acid/O_2 ratio is 3:1 when palmitic acid is present at high concentration (Boddupalli *et al.*, 1990, 1992).

The two domains of *P*-450 BM-3 can be separated by treatment with trypsin. However, in the absence of substrate the *N*-terminus of the *P*-450 domain is also cleaved at positions 9 and 15, thus losing its ability to bind substrate (Narhi & Fulco, 1987). Although the *N*-terminus of the reductase domain has been defined by protein sequencing, the *C*-terminus of the *P*-450 domain has not been determined, and from the sequence there are several potential tryptic cleavage sites in the interdomain region which could lead to heterogeneity of this domain (Ruettinger *et al.*, 1989). Similarly, the reductase domain generated by trypsin treatment appears to be heterogeneous when eluted from anion-exchange h.p.l.c. (Narhi & Fulco, 1987).

In order to begin investigations of the properties of the two domains, and in particular to compare the *P*-450 domain with the well-characterized soluble bacterial *P*-450 cam (Martinis *et al.*, 1991), with which it shares only 15% sequence identity, we decided to make genetic constructs which can overexpress both the *P*-450 and reductase domains independently of each other in *E. coli*. This paper describes the construction and expression of

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; *P*-450, cytochrome *P*-450-dependent mixed-function mono-oxygenase.

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subgenes encoding such domains, purification of the domains, characterization of the *P*-450 domain and holoenzyme using e.p.r. and resonance Raman spectroscopy, and reconstitution of mono-oxygenase activity by mixing the two individual domains.

During the preparation of this paper, reports of the generation of the two domains of *P*-450 BM-3 and their characterization by u.v.–visible spectroscopy were published (Li *et al.*, 1991; Oster *et al.*, 1991). The work described here extends the characterization of this unique system, with studies using additional spectroscopic techniques. The widespread interest in *P*-450 BM-3 reflects its potential use as a model system for studying domain interactions and intramolecular electron-transfer processes in *P*-450 systems.

EXPERIMENTAL

Bacterial strains, plasmids and bacteriophages

B. megaterium ATCC 14581, which contains the *P*-450 BM-3 gene *CYP102*, was obtained from the American Type Culture Collection, Rockville, MD, U.S.A. *E. coli* strains for plasmid and M13 propagation, and overexpression of domains were MV1190 (Δ[*lac-proAB*] *thi supE*44 Δ[*srl-recA*]306::Tn*10*[*tet*^{r}] F′[*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]) (Vieira & Messing, 1987) and XL-1 Blue (*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi relA*1 *lac*$^-$ F′ [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*(*tet*^{r})]) (Bullock *et al.*, 1987). The following vectors were used: bacteriophage M13mp18 (Yanisch-Perron *et al.*, 1985) for DNA sequencing; and plasmids pUC118 (Vieira & Messing, 1987) and ptac85 (Marsh, 1986) for overexpression.

PCR and DNA sequencing

The PCRs (Saiki *et al.*, 1988) were performed as follows. Targets were either *B. megaterium* ATCC 14581 genomic DNA (100 ng) or plasmid DNA (1 ng); 1 μg of each of the relevant pairs of oligonucleotides and 200 μM-dNTPs were added to a reaction volume of 100 μl. *Taq* DNA polymerase (2.5 units per reaction) and reaction buffer were from Promega (Chilworth Research Centre, Southampton, U.K.). Twenty-five cycles of 1 min, 94 °C, 2 min, 55 °C and 3 min, 72 °C for the genomic DNA target, and 15 cycles for the plasmid DNA target were carried out in a Perkin–Elmer Cetus Thermal Cycler.

Oligonucleotide sequences were: P9, hybridizing to positions 1525–1548 in the *CYP102* gene sequence (Reuttinger *et al.*, 1989), adding an *Eco*RI site upstream of the ribosome-binding site and P10, hybridizing to positions 2963–2933, which introduces tandem stop codons at the end of the *P*-450-domain-coding region (position 2957) and also a *Bam*HI site; P11, hybridizing to positions 4822–4800 downstream of the terminator of the *CYP102* gene adding a *Bam*HI site; and P24, hybridizing to positions 2957–2973 at the start of the reductase-domain-coding region adding a *Bam*HI site. PCR products were subcloned into M13mp18 and fully sequenced using the dideoxy chain-termination method (Sanger *et al.*, 1980) and a series of specific sequencing primers to confirm their structure.

Other molecular-biology methods

DNA manipulations, bacterial transformations and other molecular-biology methods were by standard methods (Sambrook *et al.*, 1989).

Purification of the *P*-450 domain

All purification steps were done at 4 °C unless stated otherwise. XL-1 Blue (pJM20) was the source of the *P*-450 domain, and 20–25 g of wet cells was the starting point for purification. The *P*-450 domain was purified in a similar manner to that described by Li *et al.* (1991) except that the initial ion-exchange chromatography step was performed using DEAE-Sephacel. After DEAE-Sephacel chromatography, fractions with an absorbance ratio $A_{419}/A_{280} > 0.5$ were retained and applied to Bio-Gel HTP (DNA grade) hydroxyapatite (12 cm × 3 cm column). The protein was eluted with a linear gradient of 25–450 mM-potassium phosphate, pH 6.5. Fractions containing material with $A_{419}/A_{280} > 1.2$ were pooled and applied to an f.p.l.c. Mono Q column. The protein was eluted in a linear gradient of 0–250 mM-KCl in buffer A (50 mM-Tris/HCl, pH 8.0, 1 mM-EDTA, 2 mM-2-mercaptoethanol), and pooled fractions with $A_{419}/A_{280} > 1.6$ were retained for further study.

Purification of reductase domain

XL-1 Blue (pJM27) was the source of the reductase domain. A crude extract was prepared and protein precipitating between 30% and 60% saturation with $(NH_4)_2SO_4$ contained the bulk of the reductase domain. The precipitated protein was resuspended in buffer A containing 1 mM-benzamidine plus 60 μg of phenylmethanesulphonyl fluoride (PMSF)/ml, dialysed and applied to the DEAE-Sephacel column. The column was washed, and the protein eluted with a linear gradient of 0–500 mM-KCl in buffer A (300 ml). The most intensely yellow fractions, containing *P*-450 reductase and eluted at about 130 mM-KCl, were pooled and dialysed against 2 × 11 litre of buffer B (10 mM-potassium phosphate, 0.02 mM-EDTA, pH 7.7, 0.2 mM-2-mercaptoethanol, 60 μg of PMSF/ml, 1 mM-benzamidine). The reductase domain was purified to homogeneity in a further step using 2′5′-ADP–Sepharose affinity chromatography, essentially as described by Li *et al.* (1991).

Purification of cytochrome *P*-450 BM-3

XL-1 Blue (pJM23) was the source of intact *P*-450 BM-3. Cytochrome *P*-450 BM-3 was purified in a similar manner to that described by Boddupalli *et al.* (1990), except that the final step involved 2′5′-ADP–Sepharose affinity chromatography, essentially as described by Li *et al.* (1991), rather than gel filtration.

Spectroscopy and enzyme assays

All u.v.-visible spectroscopy was performed on a Shimadzu 2100 spectrophotometer. Protein concentration was determined by the method of Lowry *et al.* (1951) with BSA as standard. Cytochrome *P*-450 concentrations were measured by the method of Omura & Sato (1964) using $\epsilon = 91\ mM^{-1} \cdot cm^{-1}$ for the reduced plus CO adduct at 450 nm. Cytochrome *c* reductase activity was measured at 30 °C by following the NADPH-dependent increase in absorbance at 550 nm, $\epsilon = 21\ mM^{-1} \cdot cm^{-1}$. The assay mixture contained 0.3 M-potassium phosphate, pH 8.0, 1.5 mg of cytochrome *c*/ml and 0.2 mM-NADPH. Fatty acid hydroxylation was measured using a spectrophotometric assay (Matson *et al.*, 1977). The assay mixture contained 0.5 mM-sodium laurate and 0.2 mM-NADPH in 0.1 M-potassium phosphate, pH 8.0, and the decrease in absorbance at 340 nm was followed using $\epsilon = 6.2\ mM^{-1} \cdot cm^{-1}$ at 30 °C. Concentration of the cytochrome *P*-450 reductase domain was estimated using $\epsilon = 21.2\ mM^{-1} \cdot cm^{-1}$ at 456 nm (Vermilion & Coon, 1978).

E.p.r. spectra were recorded with an X-band spectrometer (Bruker ER 200D, with a Datasystem) fitted with a flow cryostat (Oxford Instruments p.l.c., ESR9).

To record resonance Raman scattering, samples (170 μM-*P*-450 domain in 50 mM-Tris/1 mM-EDTA/2 mM-2-mercaptoethanol/50% glycerol) were spun in a solution cell at room temperature. A 428.1 nm laser line (10 mW nominal power) from a continuous-wave Spectra Physics 375 stilbene 3 dye laser

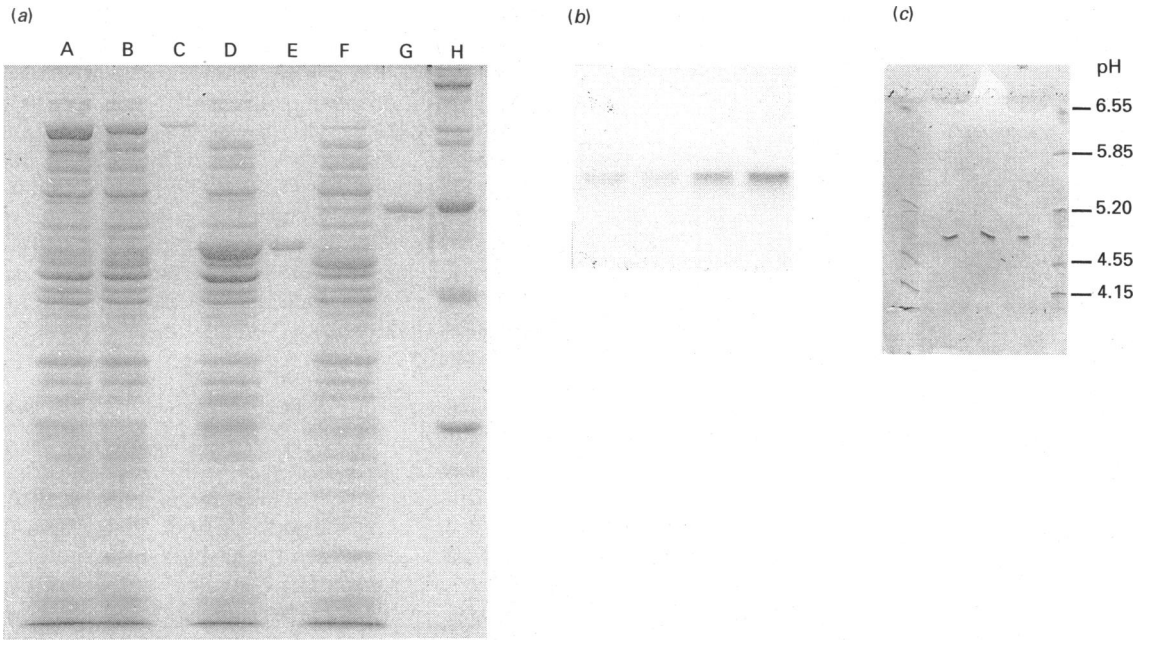

Fig. 1. Expression and purification of cytochrome *P*-450 BM-3 domains

(*a*) Overexpression of *P*-450 BM-3 (118 kDa) from plasmids pJM23 and pJM25 (lanes A and B); purified *P*-450 BM-3 (lane C); overexpression of *P*-450 domain (54 kDa) from pJM20 (lane D); purified *P*-450 domain (lane E); expression of the reductase domain (64 kDa) from pJM27 (lane F); purified reductase domain (lane G); molecular-mass markers: 205 kDa (myosin), 116 kDa (β-galactosidase), 97.4 kDa (phosphorylase *b*), 66 kDa (bovine albumin), 45 kDa (egg albumin) and 29 kDa (carbonic anhydrase) (lane H). Cells were grown overnight at 37 °C (with 50 μg of isopropyl β-thiogalactoside/ml in the case of pJM27) and whole cells analysed by SDS/PAGE. (*b*) Native PAGE and (*c*) isoelectrofocusing PAGE analyses of independent preparations of the *P*-450 domain indicate that it is a single molecular species with pI = 4.9.

pumped by a Spectra Physics 2045 argon ion laser was used to excite the haemoprotein. The spectrum was dispersed in an Anaspec modified Cary 81 scanning double monochromator (1 m focal length, 1200 grooves/mm holographic gratings blazed at 500 nm, 0.67 nm/mm linear reciprocal dispersion). The spectral resolution was 5 cm^{-1}. A cooled (−40 °C) Thorn EMI 9658R photomultiplier tube (S20 photocathode) was used as the detector with photon-counting electronics for data acquisition.

Materials

Oligonucleotides were made on an Applied Biosystems 380A DNA synthesizer and used without further purification; reagents for molecular biology were from either Gibco–BRL, Boehringer or United States Biochemicals; radiochemicals were from Amersham International; DEAE-Sephacel was from Pharmacia–LKB; Bio-Gel HTP was from Bio-Rad; the stilbene laser dye was supplied by Edinburgh Instruments (Edinburgh, Scotland, U.K.); and other reagents were from Sigma.

RESULTS AND DISCUSSION

Genes and subgenes expressing domains of *P*-450 BM-3

The *CYP102* gene expressing intact *P*-450 BM-3 and subgenes expressing either the *P*-450 domain or the reductase domain were constructed as described in the Experimental section. Genomic DNA from *B. megaterium* ATCC 14581 was isolated, a 1.5 kb fragment amplified using PCR primers P9 and P10, its sequence confirmed, and the fragment transferred into pUC118 to give pJM20. This construct contains a subgene expressing the first 472 amino acids of *P*-450 BM-3 constituting the *P*-450 domain and is expressed from the *lac* promoter in pJM20.

The entire *CYP102* gene was known to reside on a 5 kb *Bgl*II fragment (Wen & Fulco, 1987). We cloned this fragment, from genomic DNA, into pUC119 to give pJM23. As expected, pJM23 expresses intact P-450 BM-3 from its own promoter.

Amplification of pJM23 DNA using PCR primers P24 and P11 generates a 1.8 kb subgene which encodes residues 473–1049 of *P*-450 BM-3 and constitutes the reductase domain. The sequence of this fragment was confirmed, and it was cloned into the *Bam*HI site of ptac85 to give pJM27. Primer P24 introduces an ATG initiation codon at positions 2954–2956 immediately preceding the start of the reductase-domain-coding region

Purification and characterization of cytochrome *P*-450 BM-3 and its domains

The *P*-450 domain was purified and the specific content of *P*-450 haem determined to be 14.2 nmol/mg of protein; this is somewhat lower than the theoretical value of 18.6 nmol/mg based on the molecular mass. However, we believe that the purified *P*-450 domain is all holoprotein, as analysis by SDS/PAGE, native PAGE and isoelectrofocusing PAGE (pI ≈ 4.9) reveals only a single species (Fig. 1). This was substantiated by its ability to form small (20 μm diameter) needle-shaped crystals in its native form from a hanging drop over 200 mM-Tris/HCl, pH 7.62, buffer saturated with 50% $(NH_4)_2SO_4$; the crystals were too small to mount for X-ray diffraction studies (not shown).

Titration of the *P*-450 domain with substrate causes a typical Type I shift from 419 nm (low spin) to 397 nm (high spin), giving a spectral K_s for sodium palmitate of approx. 1 μM (not shown); this is similar to the K_m of *P*-450 BM-3 reported for palmitate of approx. 2 μM by Narhi & Fulco (1986).

The reductase domain was purified to homogeneity and it appears to be a single species as judged by SDS/PAGE (Fig. 1) but is susceptible to proteolysis, probably by an endogenous proteinase, giving rise to a major 45 kDa species but distinct from the product of partial trypsin digestion reported by Oster *et*

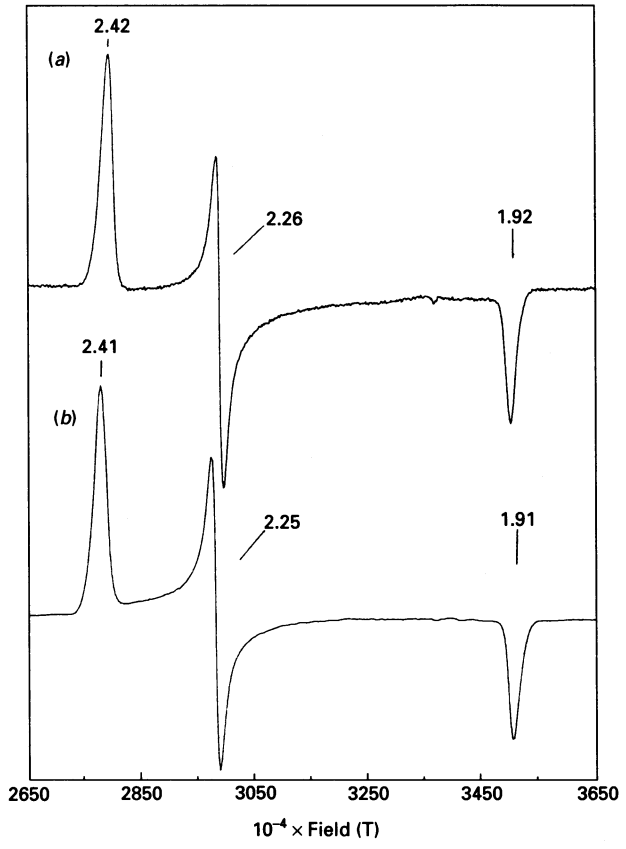

Fig. 2. E.p.r. spectra of *P*-450 domain and holoprotein

E.p.r. spectrum of the low-spin ferric *P*-450 domain (lower) and holoprotein (upper). Haem domain iron 170 μM in buffer A including 50% (v/v) glycerol and holoprotein in buffer A. Temperature of sample: 10 K. Frequency, 9.32 GHz. Modulation amplitude 10^{-3}, power 2 and 0.51 mW.

al. (1991). The cytochrome *c* reductase activity of the reductase domain is 2900 nmol/min per nmol.

The intact cytochrome *P*-450 BM-3 was purified (Fig. 1). The specific content of *P*-450 haem is 5.6 nmol/mg compared with a theoretical value of 8.4 nmol/mg; others have reported a similar discrepancy (Boddupalli *et al.*, 1990). The fatty acid (sodium laurate) hydroxylation activity was measured as 890 nmol/min per nmol and the cytochrome *c* reductase activity was 3500 nmol/min per nmol, slightly higher than the activity of the individual reductase domain. The assay mixtures contained 89 nM-*P*450 BM-3.

The u.v.–visible spectra of the oxidized, dithionite-reduced, and reduced plus CO forms of the haemoproteins, and the oxidized and dithionite-reduced reductase domain are essentially as reported previously (Narhi & Fulco, 1986; Li *et al.*, 1991; Oster *et al.*, 1991).

Reconstitution of fatty acid hydroxylase activity by mixing domains

Attempts were made to reconstitute the fatty acid hydroxylation activity associated with the intact *P*-450 BM-3 by mixing together the individual domains. Under conditions comparable with assaying *P*-450 BM-3, mixtures containing 89 nM each of *P*-450 and reductase domains when assayed failed to show any activity. Increasing the reductase domain concentration fivefold to 450 nM did not give activity. However, assay mixtures containing the *P*-450 domain at 720 nM and the reductase domain at 3600 nM gave an activity of 1.0 nmol/min per nmol of *P*-450 domain, i.e. about one thousandth of the specific activity of *P*-450 BM-3, but comparable with the specific activities found for reconstituted mammalian *P*-450s (e.g. see Wolf *et al.*, 1988; Guengerich, 1991). This is in contrast with previous attempts by others to reconstitute activity using individual domains produced by genetic engineering (Li *et al.*, 1991) or by limited trypsinolysis of intact *P*-450 BM-3 (Narhi & Fulco 1987). Our results suggest that the very high specific activity of *P*-450 BM-3 is due to the physical linkage of the *P*-450 domain to the reductase domain. The interdomain linkage may not play a passive role, but rather it may be vital for domain orientation and interaction to promote high catalytic activity.

E.p.r. spectroscopy of the haem group of *P*-450 domain and holoprotein

The X-band e.p.r. spectra of the oxidized form of the *P*-450 haem domain and of the holoprotein recorded at 10 K are shown in Fig. 2. The only signals are from the low-spin ferric haem at *g* 2.42, 2.26 and 1.92 in the haem domain and *g* 2.41, 2.25, 1.92 in the holoprotein. These *g*-values are well within the ranges reported for other *P*-450s (2.39–2.46, 2.23–2.30 and 1.90–1.93; Chevion *et al.*, 1977). Indeed, the spectra are virtually identical with those of the low-spin ferric form of *P*-450 cam (*g* 2.45, 2.26, 1.91; Dawson *et al.*, 1982; Lipscomb, 1980). The *g*-values are sensitive to the nature of the haem axial ligands and to perturbations such as the orientations of the ligands. These results show that the haem ligation in *P*-450 BM-3 is identical with that of *P*-450 cam. The presence or absence of the flavin domain does not affect the haem state in the oxidized resting state.

Since the *P*-450 domain of *P*-450 BM-3 is expressed to a high level in *E. coli* cells, the e.p.r. spectrum of the whole cells has been recorded (Fig. 3). The spectrum of the low-spin ferric *P*-450 is clearly detected. The level is sufficiently high that no other e.p.r.-active components in the cells are observed. The *g*-values recorded are 2.40, 2.24 and 1.91, almost identical with those of the purified protein. No degradation or proteolysed products are seen in cells grown as described earlier. However, we have noted that if the cells are grown on a large scale (100 litres) in a bioreactor, the cells contain more than one form of *P*-450 as judged by multiple signals in the whole-cell e.p.r. spectra.

Resonance Raman spectroscopic analysis of *P*-450 domain

The resonance Raman spectra of the substrate-bound and substrate-free *P*-450 domains have been recorded (Fig. 4). The substrate-free *P*-450 haem iron exists in the ferric (Fe^{3+}) state in the presence of aerobic O_2. This is confirmed by the position of ν_4 (oxidation state marker) at 1371 cm^{-1}. The position of the spin-state-sensitive bands attributable to ν_2 and ν_3 at 1585 cm^{-1} and 1500 cm^{-1} respectively indicate that the protein haem is in a low-spin-state configuration. The spin-state/co-ordination-state marker band due to ν_{10} at 1639 cm^{-1} indicates that the haem is in a six-co-ordinate low-spin state. The peak alongside that due to ν_{10} may be attributed to the stretching of the peripheral vinyl groups of the protoporphyrin IX haem groups. Interestingly, the relative intensity of this band is somewhat greater than that observed for *P*-450 cam in the reported Soret excited resonance Raman spectrum (Champion *et al.*, 1978; Bangcharoenpaurpong *et al.*, 1987). Although there is an approx. 10 nm difference in exciting line wavelength [417.5 nm used by Bangcharoenpaurpong *et al.* (1987) compared with 428.1 nm in this work], the spectral differences observed may be attributable to subtle differences between the structures of the haem prosthetic groups of *P*-450 BM-3 and *P*-450 cam. In particular, this may reflect the different amino acid environments between *P*-450 BM-3 and *P*-

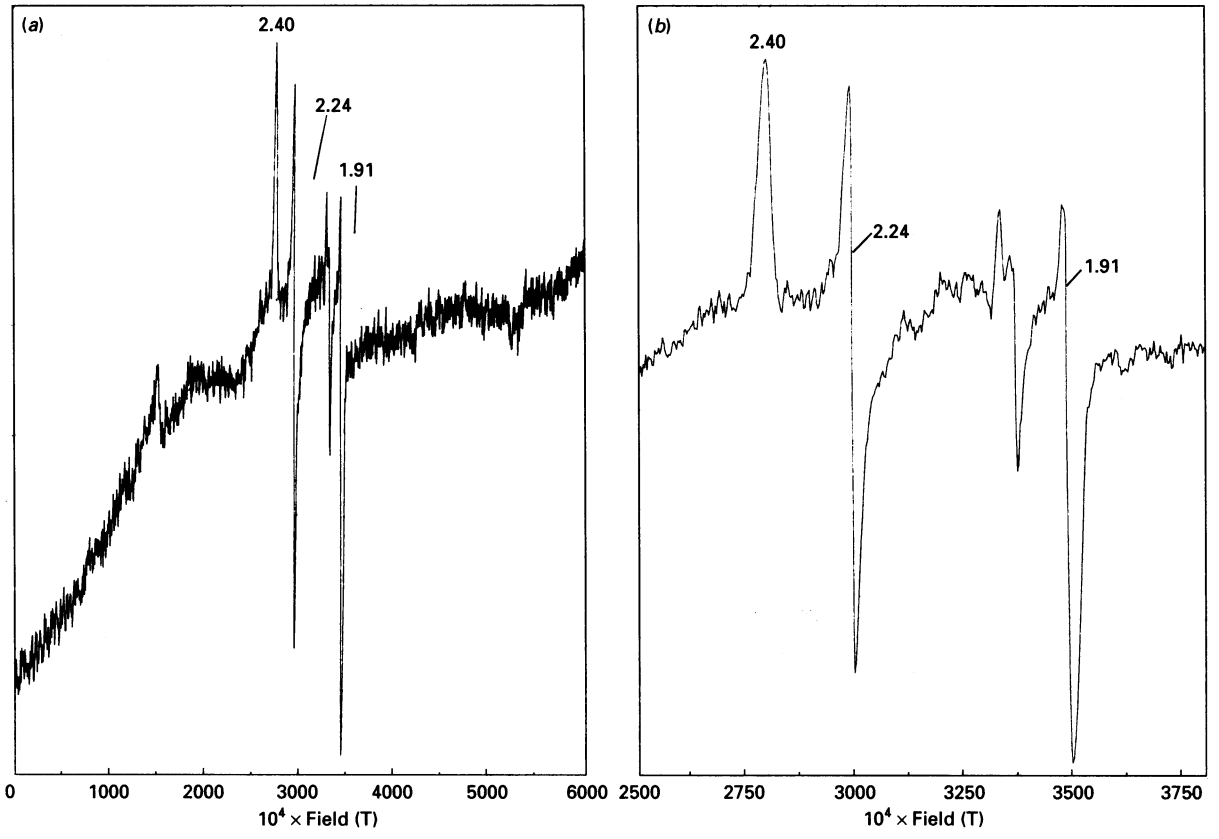

Fig. 3. E.p.r. spectrum of whole cells of *E. coli* XL-1 Blue (pJM20)

Cells were washed in 10 mM-EDTA/50 mM-Tris/HCl, pH 7.5, and spun down. This was repeated in 1 mM-EDTA using the same buffer. Cells suspended in buffer A, were spun in an e.p.r. tube. Total amount of cells 0.39 g wet weight. Conditions of measurement as for Fig. 2. (*a*) Wide field scan, (*b*) expanded field scale.

450 cam about the periphery of the haem group which will arise as a result of the low sequence similarity (15% identity).

Assignment of the band at 350 cm^{-1} may be made to Fe-S_{Cys}. Additional low-frequency features are observed between 400 and 500 cm^{-1} and the band at 428 cm^{-1} may be attributable to Fe-O_{axial} stretching. The bands at 678 cm^{-1} and 756 cm^{-1} are assigned to ν_7 and ν_{15} respectively. The latter mode has been proposed to be the Jahn–Teller active vibration for metalloporphyrins and metallophthalocyanines (Bovill *et al.*, 1992).

Addition of palmitate (1 mM final concentration) to the *P*-450 domain produced the high-frequency spectrum shown in Fig. 3(*b*) and reveals a substrate-induced protein haem low-to-high spin state change. This is indicated by the broad bands attributable to ν_2 and ν_{10} at 1575 cm^{-1} and 1625 cm^{-1} respectively. These lower frequencies, as compared with those observed with the substrate-free protein, can be correlated with five-coordinate high-spin-state haem.

Conclusions

The high-level expression of *P*-450 BM-3 and its two functional domains in *E. coli*, and their relatively facile purification, allows large quantities of protein to be produced for biophysical and biochemical studies. The similarity of the *P*-450 domain to members of *P*-450 family 4, and the reductase domain to eukaryotic *P*-450 reductases, means that the holoprotein and the individual domains provide excellent models for the microsomal *P*-450 system. In particular, *P*-450 BM-3 can now be used as an alternative model to that of *P*-450 cam. The e.p.r. studies described in this work indicate that the haem ligation of the *P*-450 domain, and *P*-450 BM-3, is identical with *P*-450 cam. Interestingly, the presence of the reductase domain in the intact *P*-450 BM-3 does not modify the e.p.r. spectrum, indicating that interaction of the two domains does not change the haem ligation state. Further studies of the haem environment and ligation state of the *P*-450 domain and *P*-450 BM-3 will be made using near-i.r. magnetic c.d. spectroscopy.

Comparison of the resonance Raman spectrum of the substrate-free *P*-450 domain with the equivalent spectrum of *P*-450 cam suggests that the peripheral interactions of the protein with the protoporphyrin IX, especially the vinyl groups, is somewhat different from that in *P*-450 cam. Although the amino acid sequence similarity between the *P*-450 domain and *P*-450 cam is highest around the conserved cysteine ligand to the haem iron, there is still considerable variation in residues which may contact the haem and which may cause subtle alterations of the haem electronic structure. Further studies of the *P*-450 domain and intact *P*-450 BM-3, including a comparison of the resonance Raman and surface-enhanced resonance Raman spectra [Rospendowski *et al.* (1991) and references therein], will help clarify this point. Fluorescence interference obscures the resonance Raman spectra of the intact *P*-450 BM-3 under the existing experimental conditions, but low-temperature studies may help.

The observations that the catalytic rate of a mixture of the two individual domains is comparable with those found for reconstituted mammalian microsomal *P*-450s suggests that the covalent linkage of the two domains is the key to the high catalytic rate in the intact *P*-450 BM-3. Future work in this direction should be aimed at making fusion proteins containing either the *P*-450 domain of *P*-450 BM-3 and eukaryotic

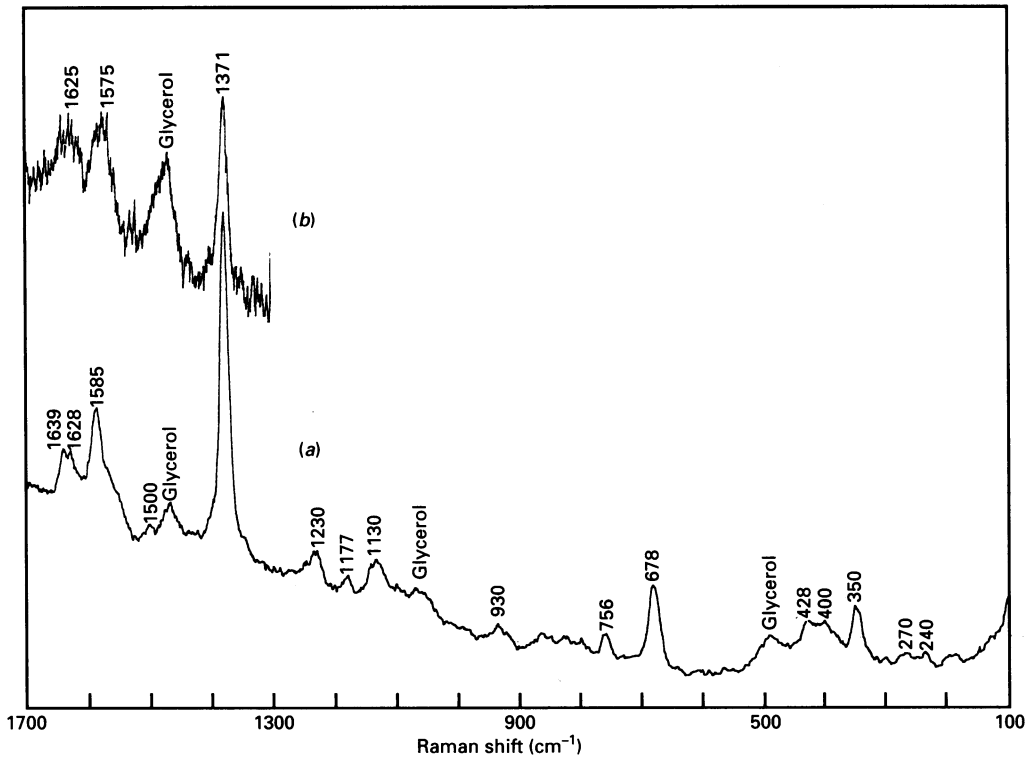

Fig. 4. Resonance Raman spectrum of *P*-450 domain

The resonance Raman spectra of (*a*) the low-spin ferric substrate-free *P*-450 domain and (*b*) the high-spin ferric substrate-bound *P*-450 domain. A 428.1 nm laser line (10 mW nominal power) was used to excite the haemoprotein. The protein samples were (*a*) 170 μM *P*-450 domain in buffer A including 50% (v/v) glycerol and (*b*) an equal volume of this mixed with palmitate dissolved in 50 mM-potassium carbonate, pH 8.0, to give a final concentration of substrate of about 1 mM. The salient features of the spectra are described in the Results and Discussion section. The deterioration of the signal-to-noise ratio for the substrate-bound versus substrate-free spectrum can be rationalized on the basis of the hypsochromic shift in the Soret band λ_{max} = 419–397 nm on addition of substrate. The 428.1 nm laser excitation line is further off-resonance with respect to the high-spin-state haem electronic Soret transition. In the overtone and combination band region (1700–3500 cm^{-1}), only barely detectable features were observed (not shown). The peaks due to glycerol are marked.

reductases, or mammalian microsomal *P*-450s with the reductase domain of *P*-450 BM-3 to determine whether such proteins can be expressed in *E. coli* and an increase in catalytic activity can be achieved.

The work carried out at Glasgow was supported by SERC grant GR/F76046 to J.S.M. Support of the MRI of SERC is gratefully acknowledged for the work carried out at UEA, and B.N.R. receives support from SERC.

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Received 15 April 1992/8 June 1992; accepted 23 June 1992