Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 342 (2006) 191–196
BBRC
www.elsevier.com/locate/ybbrc

Cytochrome P450 enzymes from the metabolically diverse bacterium *Rhodopseudomonas palustris*

Stephen G. Bell [a,*], Nicola Hoskins [a], Feng Xu [b], Domenico Caprotti [a], Zihe Rao [b], Luet-Lok Wong [a,*]

[a] *Department of Chemistry, University of Oxford, Inorganic Chemistry Laboratory, South Parks Road, Oxford, OX1 3QR, UK*
[b] *Laboratory of Structural Biology, Department of Biological Science and Technology and MOE Laboratory of Protein Science, Tsinghua University, Beijing 100084, China*

Received 5 January 2006
Available online 3 February 2006

Abstract

Four (CYP195A2, CYP199A2, CYP203A1, and CYP153A5) of the seven P450 enzymes, and palustrisredoxin A, a ferredoxin associated with CYP199A2, from the metabolically diverse bacterium *Rhodopseudomonas palustris* have been expressed and purified. A range of substituted benzenes, phenols, benzaldehydes, and benzoic acids was shown to bind to the four P450 enzymes. Monooxygenase activity of CYP199A2 was reconstituted with palustrisredoxin A and putidaredoxin reductase of the P450cam system from *Pseudomonas putida*. We found that 4-ethylbenzoate and 4-methoxybenzoate were oxidized to single products, and 4-methoxybenzoate was demethylated to form 4-hydroxybenzoate. Crystals of substrate-free CYP199A2 which diffracted to ~2.0 Å have been obtained.

Keywords: *Rhodopseudomonas palustris*; P450 enzymes; Substrate specificity; Monooxygenases; Electron transfer

Rhodopseudomonas palustris is a common soil and water bacterium regarded as one of the most metabolically versatile bacteria ever described. It can grow aerobically or anaerobically, convert carbon dioxide into biomass, nitrogen to ammonia, produce hydrogen gas, and break down organic compounds such as fatty acids, dicarboxylic acids, and plant-derived aromatic acids [1–4]. The enzymes and biochemical pathways that endow this organism with its metabolic diversity are of great scientific interest and potentially a rich source of novel enzymes for biotechnological applications. The *R. palustris* genome sequence was determined recently, enabling structural and functional genomic investigations on this fascinating organism [5].

Cytochrome P450 (CYP) enzymes are heme-dependent monooxygenases that catalyze the insertion of an oxygen atom from atmospheric dioxygen into C–H bonds in many classes of organic compounds [6,7]. The physiological role of P450 enzymes includes biosynthesis of endogenous compounds such as steroids, hormones, and secondary metabolites, fatty acid oxidation, and xenobiotic and especially drug metabolism [8–10]. The reactions are often substrate specific and show high selectivity of product formation. The active site architecture and protein dynamics that control P450 activity are of fundamental scientific interest and their detailed understanding has implications in biotechnological applications. P450 enzymes have been implicated in the degradation of organic compounds such as tri-*n*-butylphosphate by *R. palustris* [11]. Identification of the substrates and electron transfer mechanisms of P450 enzymes from a metabolically diverse organism such as *R. palustris* will provide new systems for detailed study and may add to the repertoire of enzymes for applications.

The *R. palustris* genome (http://genome.ornl.gov/microbial/rpal/) contains seven P450 (CYP) genes. No analogues to the self-sufficient Class III or IV P450 enzymes such as CYP102A1 or $P450_{RhF}$ were found [12–14]. Of the seven

* Corresponding authors. Fax: +44 1865 272690.
E-mail addresses: stephen.bell@chem.ox.ac.uk (S.G. Bell), luet.wong@chem.ox.ac.uk (L.-L. Wong).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.01.133

Table 1
Rhodopseudomonas palustris P450 (CYP) genes and the potential electron transfer co-factor proteins

P450 enzyme	Associated electron transfer protein
RPA1613 (CYP153A5)	
RPA1732 (CYP194A2)	RPA1731 (Ferredoxin 3Fe–4S)
RPA3778 (CYP195A2)	
RPA0785 (CYP196A2)	
RPA1871 (CYP199A2)	RPA1872 (Ferredoxin 2Fe–2S)
RPA0421 (CYP201A2)	
RPA1009 (CYP203A1)	

enzymes (Table 1) two have associated ferredoxin-like proteins which might be the physiological electron transfer co-factor proteins, indicative of Class I, three-component electron-transfer chains commonly found in bacteria [15]. None of the CYP genes, however, have an associated ferredoxin reductase such as that found for the P450cam system from *Pseudomonas putida* [16].

The *R. palustris* P450 enzymes belong to different bacterial P450 families, suggesting a different substrate or range of substrates for each enzyme. CYP153A5 is related to CYP153A1 from *Acinetobacter calcoaceticus* and P450 enzymes in *Mycobacterium* and *Sphingomonas* sp. [17,18]. Most members of the CYP153 family oxidize C_5–C_{12} *n*-alkanes but some have also been shown to oxidize limonene and *N*-benzylpyrrolidine [19]. CYP203A1 is the first example of a P450 enzyme for this family. A recent report showed that a CYP203 enzyme from an environmental metagenome library oxidized 4-hydroxybenzoate to protocatechuate (3,4-dihydroxybenzoate) [20]. The other five *R. palustris* CYP genes are all similar to ones found in the bacterium *Bradyrhizobium japonicum* but none has been expressed and their substrate specificity and function are not known. Early studies on *B. japonicum* lead to suggestions that some of the P450 enzymes might have novel functions such as terminal oxidases, peroxidases, or membrane bound oxygen carriers in an oxidative phosphorylation pathway [21,22].

Here we report the expression and preliminary characterization of some of the *R. palustris* P450 enzymes.

Materials and methods

General. Enzymes for molecular biology were from New England Biolabs, UK. KOD polymerase (Merck Biosciences) was used for the PCR steps. Buffer components were from Anachem, UK, and general reagents were from Sigma/Aldrich or Merck, UK. The substrates tested for binding to the P450 enzymes were from Sigma–Aldrich, Lancaster and Acros Organics (all in UK). NADH and isopropyl-β-D-thiogalactopyranoside (IPTG) were from Roche Diagnostics, UK. UV/Vis spectra and spectroscopic activity assays were recorded at 30 °C on a Varian CARY 1E spectrophotometer. Electrospray protein mass spectrometry experiments were carried out on a Micromass platform II instrument. Gas–liquid chromatography (GC) analyses were carried out on a Thermo-Finnigan 8000Top instrument equipped with a flame-ionization detector (FID) using a DB-1 fused silica capillary column (0.5 mm × 7.5 m) and helium as the carrier gas.

Enzymes and molecular biology. General DNA manipulations and microbiological experiments were carried out by standard methods [23]. The pCWori+ vector was from Richard Dalquist (University of Oregon, Eugene, Oregon, USA) and Roland Wolf (University of Dundee, UK). Proteins were stored at −20 °C in 50 mM Tris, pH 7.4, containing 50% v/v glycerol. Glycerol was removed immediately before use by gel-filtration on a 5-mL PD-10 column (Amersham-Pharmacia Biotech) by eluting with 50 mM Tris, pH 7.4.

The genomic DNA of *R. palustris* (ATCC BAA-98) was obtained from ATCC-LGC Promochem, UK. The genes encoding the seven P450 enzymes and the putative 2Fe–2S (RPA1872) and 3Fe–4S (RPA1731) ferredoxins were amplified by PCR. The oligonucleotide primers used for amplification and cloning of the genes are listed in the Supplementary Material. The CYP genes were amplified by 25 cycles of strand separation at 95 °C for 1 min followed by annealing at 42 °C and extension at 68 °C for 2 min. The genes encoding the putative ferredoxins were amplified using a shorter extension time of 1 min. The amplified genes were cloned into the expression vectors pCWori+, pET26 or pET28 (Merck Biosciences) by using appropriate restriction sites introduced at the PCR step. All the amplified genes were fully sequenced by automated DNA sequencing on an ABI 377XL Prism DNA sequencer by the DNA sequencing facility at the Department of Biochemistry, University of Oxford.

Protein expression and purification. The putative ferredoxin encoded by the RPA1872 gene was expressed in *E. coli* DH5α harboring a pCWori+ plasmid containing the gene. The expressed protein was purified by the method described for putidaredoxin from *P. putida* [16].

All the P450 enzymes were expressed from a T7 promoter using the pET26 or PET28 vector in *E. coli* JM109(DE3) or BL21(DE3) as the heterologous host. A single colony was inoculated into 200 mL Luria–Bertani broth (LB_{kan}) containing 30 μg/mL kanamycin and grown at 37 °C overnight. This culture was then inoculated at a ratio of 15 mL/L into 6 × 1-L of LB_{kan} medium containing 0.4% (v/v) glycerol, and grown at 37 °C for 4–6 h to OD_{600} ~1. Recombinant protein expression was induced by the addition of 0.4 mM IPTG (from a 1 M stock in water) and the incubator temperature was lowered to 30 °C. The culture was then grown for at least another 6 h. The red cell pellet harvested from the culture by centrifugation was resuspended in 200 mL buffer T (50 mM Tris, pH 7.4, 2 mM DTT) and the cells lysed by sonication. Cell debris were removed by centrifugation at 37,000*g* for 30 min at 4 °C and the supernatant subjected to ammonium sulfate fractionation. The material precipitated at <20% saturation was white to light brown in color and discarded. The P450 enzymes were precipitated in the 40% saturation fraction. The red P450-containing pellet was resuspended in buffer T and desalted using a Sephadex G-25 column (200 × 40 mm) that had been pre-equilibrated with buffer T. The resultant red eluent was loaded onto a DEAE Fastflow Sepharose column (200 × 50 mm) and the P450 protein eluted using a linear salt gradient of 0–150 mM KCl in buffer T developed over eight column bed-volumes at a flow rate of 8 mL/min. The red P450 fractions were collected, concentrated by ultrafiltration, and desalted using a Sephadex G-25 column. The final purification step was anion-exchange chromatography using a Source-Q column (120 × 26 mm; Amersham-Pharmacia Biotech), and the protein was eluted using a linear gradient of 20–50 mM KCl in buffer T developed over 20 column bed-volumes at a flow rate of 8 mL/min. All fractions with A_{418}/A_{280} >1.2 (except for CYP203A1 which showed a hyperporphyrin spectrum in the presence of DTT with a split Soret at 375 and 455 nm and so the ratio used was A_{375}/A_{280} >0.6) were combined and concentrated by ultrafiltration to *ca.* 50 μM. Protein concentrations were estimated using $\varepsilon_{448} = 120\ mM^{-1}cm^{-1}$ for the $Fe^{II}(CO)$ complexes. An equal volume of glycerol was added, the solution filtered through a 0.22-μm sterile syringe filter and stored at −20 °C.

Mass spectrometry. Salts were removed from proteins by elution through a PD-10 column with 50 mM Tris buffer and the eluted proteins quantitated by UV/Vis spectroscopy. The buffer was exchanged with double-distilled water by successive concentration and dilution using a Millipore Amicon Ultra-4 15 Centricon at 7000*g*, 4 °C, until the final concentration of Tris was below *ca.* 50 μM. The concentrated protein solution was diluted to a final concentration of between 10 and 20 μM in a 1:1 solution of H_2O and MeCN containing 0.2% formic acid. Samples

were analyzed using a Micromass Platform II instrument coupled to an Agilent 1100 HPLC, and 10 μL was injected at a rate of 0.05 mL/min. The mass spectrometer is capable of unit mass resolution and calibrated between 600 and 2500 Da using myoglobin. The spectra were acquired in MCA with a cone voltage ramp between 30 and 100 V and a capillary voltage of 3 kV.

Spin-state shift and substrate binding constant determinations. The spin-state shift and substrate binding constants were determined at 30 °C by following the spectral changes between 350 and 450 nm on addition of increasing amounts of titrant [24]. The protein was equilibrated into 50 mM Tris, pH 7.4, using a PD-10 (Amersham-Pharmacia Biotech) gel-filtration column and diluted to *ca.* 5 μM. Spin-state shifts upon substrate binding were assayed by adding 1-μL aliquots of a 100 mM stock of substrate (typically in ethanol) to 500 μL of the P450 enzyme solution and recording the spectrum. The high spin heme content was estimated (to approximately ±5%) by comparison with a set of spectra generated from the sum of the appropriate percentages of the spectra of low and high spin forms of cytochrome P450cam (CYP101A1) (Table 2).

In substrate binding titrations, difference spectra were recorded (using the substrate-free form of the enzyme as the reference spectrum) after the addition of small (μL) aliquots of 2, 5, and 10 mM stock solutions of the substrate to 1500 μL of *ca.* 5 μM P450 enzyme so as to keep any volume change negligible (typically <10 μL total added). The binding constants were obtained by fitting the peak-to-trough difference in absorbance against substrate concentration to a Michaelis–Menten type hyperbolic function using the Origin 7 program (Origin Labs).

NADH turnover rate and metabolite determinations. Incubation mixtures (1.5 mL) contained 50 mM Tris buffer, pH 7.4, 0.5 μM P450, 5 μM ferredoxin, 0.5 μM putidaredoxin reductase, and 100 μg/mL bovine liver catalase. The mixtures were oxygenated and then equilibrated at 30 °C for 2 min. Substrate was added as a 100 mM stock solution in ethanol to a final concentration of 1 mM. NADH was added to *ca.* 320 μM (final $A_{340} = 2.00$) and the absorbance at 340 nm was monitored. The rate of NADH consumption was calculated using $\varepsilon_{340} = 6.22\ \mathrm{mM^{-1}\ cm^{-1}}$.

After all the NADH had been consumed in an incubation reaction, 3 μL of trifluoroacetic acid was added to the mixture. Control experiments showed that the final pH of the resultant mixture dropped to 1. Organics were then extracted with 3 × 0.5 mL of $CHCl_3$ and the layers separated by centrifugation at 4000*g*, 4 °C, for 20 min. The organic layer was dried over $MgSO_4$ and evaporated under a slow stream of nitrogen. The substrates and products were dissolved in CH_3CN and analyzed on a DB-1 fused silica column. The injector was held at 250 °C and the detector at 250 °C. The column temperature was held at 60 °C for 1 min and then increased at 15 °C/min to 150 °C. The retention times were: 4-methoxybenzoate 4.78 min; 4-hydroxybenzoate 5 min.

The concentration of hydrogen peroxide formed by uncoupling during NADH oxidation was determined by the horseradish peroxidase/phenol/4-aminoantipyrine assay. The NADH incubation was repeated without catalase. A 400-μL aliquot of the incubation mixture was taken, and 100 μL of 100 mM phenol in 50 mM Tris, pH 7.4, 100 μL of 10 mM 4-AP, and 200 μL of buffer were added. The change in absorbance at 510 nm was then measured on addition of 1 μL HRP solution (20 mg/mL). These A_{510} values were compared against control assays containing known concentration of hydrogen peroxide.

Crystallization of CYP199A2. Crystals of the CYP199A2 enzyme were obtained at 291 K by the hanging drop vapor diffusion method. Immediately prior to crystallization experiments the protein was further purified by size exclusion chromatography on a Superdex 200 (Amersham-Pharmacia Biotech) column (10 mm × 30 cm), eluting with 50 mM HEPES, pH 7.4, 50 mM KCl, at a flow rate of 0.8 mL(min)$^{-1}$. The purified protein was dialyzed against crystallization buffer (20 mM Hepes, pH 7.4, 200 mM KCl) and concentrated by ultrafiltration to 60 mg(mL)$^{-1}$. The protein solution (1.5 μL) was mixed with 1.5 μL of reservoir solution (and an additional 0.3 μL of additive solution in further screens) and the mixture was equilibrated against 200 μL reservoir solution at 289 K. Initial crystallization conditions were screened using Crystal Screen reagent kits (Hampton Research). The conditions of 30% PEG4000, 0.1 M sodium citrate, pH 5.6, 20% isopropanol, yielded needle-like crystals, and hence were further optimized by variation of precipitant, protein concentration, buffer pH, and Additive Screen kits (Hampton Research). Good-quality, well diffracting crystals could be obtained in one week from 15% PEG4000, 0.1 M sodium citrate, pH 5.6, 20% isopropanol with 4% v/v *t*-butanol additive.

Results and discussion

Expression and purification of P450 enzymes

All seven CYP genes from *R. palustris* were readily amplified by PCR and cloned into standard expression vectors. The determined nucleotide sequences of the cloned genes were in complete agreement with those in the genome database. Of the seven genes, four (CYP153A5, CYP195A2, CYP199A2, and CYP203A1) were over-expressed in soluble form in *Escherichia coli* and purified in reasonable yields (>10 mg/L) by ammonium sulfate fractionation followed by two anion exchange chromatography steps. CYP201A2 was expressed at low levels and could not be isolated in a pure form whilst CYP196A2 and CYP194A2 have yet to be expressed in soluble form.

The four P450 enzymes were isolated in their low-spin ferric forms, with Soret maxima at *ca.* 418 nm and all showed the characteristic 448–450 nm absorption for the $Fe^{II}(CO)$ complex with no evidence of the inactive P420 form. Dithiothreitol (DTT) is known to bind to P450 enzymes to cause a split Soret spectrum which offers a useful screen for substrate binding by following the disappear-

Table 2
Rhodopseudomonas palustris P450 enzymes and example of substrates that induce a Type I spin-state shift (estimated to within∼5%)

CYP195A2 (% HS heme)	CYP199A2 (% HS heme)	CYP203A1 (% HS heme)
Salicylic acid (50)	L-Perillic acid (>95)	L-Perillic acid (75)
4-Fluorosalicylic acid (75)	4-Chlorobenzoic acid (80)	1,2,4-Trichlorobenzene (80)
5-Fluorosalicylic acid (20)	3-Chlorobenzoic acid (10)	Pentachlorobenzene (80)
6-Fluorosalicylic acid (20)	4-Chlorobenzaldehyde (90)	1,2-Dichlorobenzene (50)
3-Chlorosalicylic acid (70)	Benzoic acid (35)	1,4-Dichlorobenzene (40)
4-Chlorosalicylic acid (85)	4-Methylbenzoic acid (80)	2,4,5-Trichlorophenol (>95)
5-Chlorosalicylic acid (20)	4-Ethylbenzoic acid (>95)	3,4-Dichlorophenol (85)
3-Methylsalicylic acid (80)	4-Isopropylbenzoic acid (>95)	2,3,5-Trichlorobenzoic acid (85)
4-Methylsalicylic acid (75)	4-*t*-Butylbenzoic acid (>95)	Benzoic acid (10)
	4-Methylsalicylic acid (>95)	4-Hydroxybenzoic acid (50)
	4-Methoxybenzoic acid (>95)	

ance of the characteristic split spectrum. However, only CYP203A1 showed the split Soret spectrum in the presence of DTT.

Substrate binding

The substrate specificity of the four P450 enzymes was investigated by screening representative examples of classes of compounds that were known to be metabolized by *R. palustris* or oxidized by other P450 enzymes. These included cyclic mono- and sesqui-terpenoids, short and medium chain (C_6–C_{12}) linear alkanes, trialkylphosphites and phosphates, benzene and polycyclic aromatic hydrocarbons, fatty acids, substituted phenols, substituted benzoic acids, polychlorinated benzenes, and steroidal compounds. Substrate binding was assayed by the classical Type I spectrum, with a shift in the Soret band from 418 nm in the substrate-free form to 390 nm for the high spin form when a substrate was bound in close proximity to the heme to displace the water bound to the iron.

None of the test substrates induced a spin-state shift for CYP153A5. This was surprising in view of the alkane oxidation activity of all the other enzymes in the CYP153 family. On the other hand, CYP195A2, CYP199A2, and CYP203A1 showed large Type I shift with a variety of compounds (Table 2). CYP203A1 was shown to bind a broad range of substrates, in particular substituted aromatic compounds. Interestingly, chlorinated benzenes, phenols, and benzoic acids were all substrates despite their different polarities, and the spin-state shift increased with the degree of substitution. CYP195A2 appeared to be highly specific for substituted salicylic acids. CYP199A2 showed the highest shifts with benzoic acids and benzaldehydes containing a substituent at the 4-position, while substituents at the 2- or 3-position tended to weaken binding, e.g., benzoic acid (35% high spin heme), 4-chlorobenzoic acid (80%), 2-chlorobenzoic acid (<5%), 3-chlorobenzoic acid (10%), 2,4-dichlorobenzoic acid (30%) 3,4-dichlorobenzoic acid (20%), 2,4,6-trichlorobenzoic acid (<5%), and 4-chlorosalicylic acid (75%). The requirement of an aromatic ring for binding was investigated with pentanoic, hexanoic, octanoic, decanoic, myristic, and palmitic acids but none of these showed any evidence of binding to CYP199A2 or CYP203A1. However, the alicyclic perillic acid, although not 1-cyclohexene carboxylic acid, induced similar spin-state shifts to benzoic acids, suggesting that a cyclic structure with a substituent in the 4-position might be a requirement of these two enzymes.

Table 3
Substrate binding and catalytic properties of CYP199A2 with some identified substrates

Substrate	% HS	Dissociation constant, *K*d (μM)	K_d (μM) with 200 mM KCl	NADH oxidation rate
4-Ethylbenzoic acid	>95	1.44	3.99	356
4-Isopropylbenzoic acid	>95	2.02	—	182
4-*t*-Butylbenzoic acid	>90	22.8	—	87
4-Methoxybenzoate	>95	—	—	174
4-Ethylbenzaldehyde	>95	54.0	53.5	—
4-Isopropylbenzaldehyde	>95	56.6	108.0	—

The estimated high-spin heme contents are to ±5%. The other data are averages of at least three experiments, with standard deviations typically ~5% of the mean. The NADH turnover rates are given in nmol per nmol-P450 per min.

The substrate binding constants of CYP199A2 (Table 3) revealed that substituted benzoic acids were bound much more tightly than substituted benzaldehydes. Increasing the size of the substituent at the 4-position weakened substrate binding. The binding constants (~1 μM) for 4-ethyl and 4-isopropylbenzoic acids were comparable to that for camphor binding by P450cam. Unlike P450cam, however, there was no tightening of substrate binding in the presence of potassium ions [25,26].

Activity reconstitution

There was no evidence of any self-sufficient Class III or IV P450 enzyme in the *R. palustris* genome. Two putative Fe–S electron transfer proteins are associated with two of the P450 enzymes. One putidaredoxin-type 2Fe–2S protein (RPA1872) is just downstream of the RPA1871 gene that encodes CYP199A2 and a 3Fe–4S protein (RPA1731) is associated with CYP194A2 (RPA1730). This is indicative of Class I P450 electron transfer chains. We were unable to express CYP194A2 or RPA1731 at satisfactory levels for further investigation.

The RPA1872 gene was readily expressed in *E. coli* but the brown protein could only be isolated in low yield (~2 mg/L). Initial investigations suggested that the protein was not as soluble as putidaredoxin of the CYP101A1 system. The electrospray mass spectrum showed a molecular mass of 11,038 ± 2 Da, in excellent agreement with the mass of 11,037 Da predicted from the nucleotide sequence. The protein showed very similar spectral properties (Fig. 1) to putidaredoxin. The data suggested that the RPA1872 gene encoded a 2Fe–2S protein, which we have tentatively named palustrisredoxin A.

In order to reconstitute P450 monooxygenase activity for the CYP199A2 system, a ferredoxin reductase analogous to putidaredoxin reductase of the P450cam system was required to mediate electron transfer from NADH to the 2Fe–2S redoxin. Although there were a number of putative flavoprotein reductase genes in the *R. palustris* genome, none was in the vicinity of RPA1871. We therefore attempted to reconstitute CYP199A2 enzymatic activity with putidaredoxin reductase. In the presence of putidaredoxin reductase and palustrisredoxin A, and using 4-ethylbenzoic acid as the substrate, CYP199A2 oxidized NADH at a rate of 356 nmol (nmol P450)$^{-1}$ (min)$^{-1}$. No hydrogen peroxide (which would arise from uncoupling of NADH turnover from product formation) was detected in the reaction mixture. The results indicated that palustrisredoxin A was the natural electron transfer cofactor protein for CYP199A2 and catalytic activity was

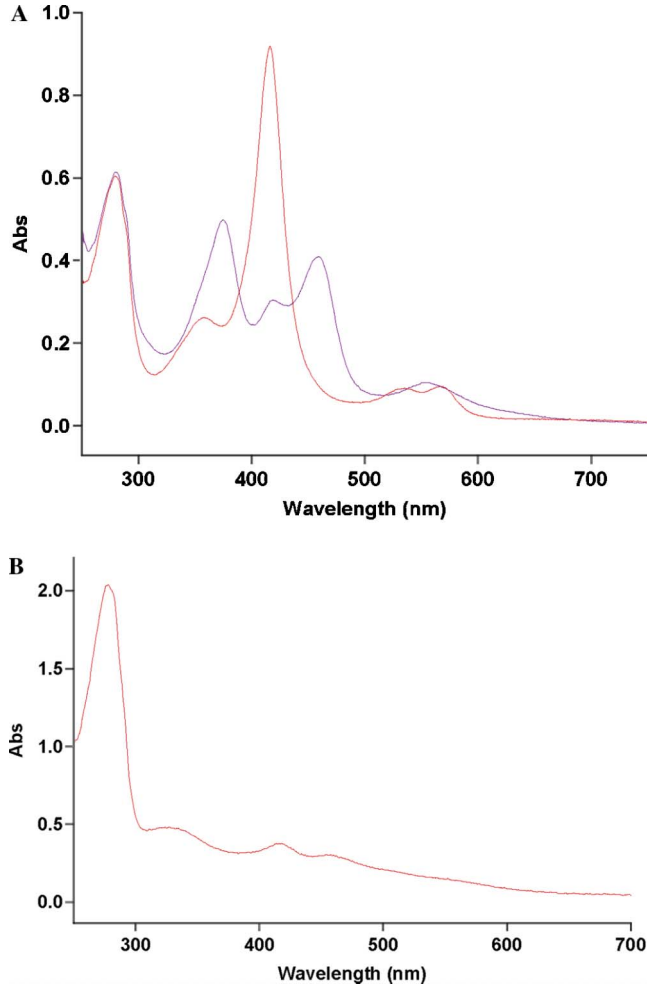

Fig. 1. Electronic spectra of (A) substrate-free (—) and DTT bound CYP203A1(—), and (B) palustrisredoxin A (RPA1872).

successfully reconstituted. Although 4-ethylbenzoic acid might not be the "natural" substrate for the enzyme, the turnover activity was similar to the *ca.* 1000 min^{-1} camphor oxidation activity of P450cam. The NADH oxidation rate was measured with other substrates (data not shown). In general, the activity decreased as the strength of the substrate binding decreased. In the absence of substrate, the NADH oxidation activity of the CYP199A2 system (7.2 min^{-1}) was 50-fold lower than observed with 4-ethylbenzoic acid. Hence CYP199A2 is also subjected to "gating" of the catalytic cycle by substrate binding commonly observed for bacterial and some eukaryotic P450 enzymes [9,27].

The NADH oxidation rate was very low (11.3 min^{-1}) when putidaredoxin and putidaredoxin reductase were used with CYP199A2 and 4-ethylbenzoic acid as the substrate. Similarly when putidaredoxin in the P450cam system was replaced with palustrisredoxin A, the NADH oxidation rate of P450cam with camphor was reduced drastically (4.5 min^{-1}). This low activity was probably due to the absence of a terminal tryptophan residue in palustrisredoxin A, which had been shown to be important for electron transfer from putidaredoxin to P450cam [28], while

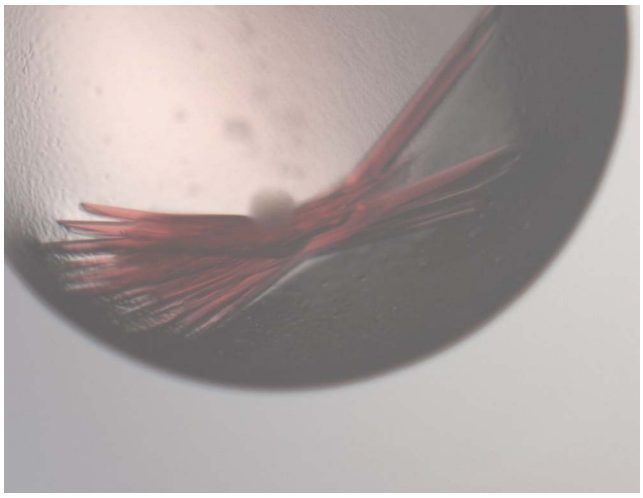

Fig. 2. Crystals of CYP199A2.

the presence of this residue interfered with binding and electron transfer from putidaredoxin to CYP199A2. There was no NADH turnover when putidaredoxin reductase and palustrisredoxin A (or putidaredoxin) were used with CYP203A1 or CYP195A2 for any of the identified substrates for these two enzymes. As no substrate has yet been found for CYP153A5 we were not able to test electron transfer to this enzyme.

Putidaredoxin reductase and palustrisredoxin A were used to examine the oxidation of 4-ethylbenzoic acid and 4-methoxybenzoic acid. After acidification with trifluoroacetic acid organics were extracted into chloroform. Gas chromatography analysis showed that both 4-ethylbenzoic acid and 4-methoxybenzoic acid were oxidized to single products. The oxidation product of 4-ethylbenzoic acid is being characterized. The product from 4-methoxybenzoic acid oxidation was identified as 4-hydroxybenzoate by co-elution with an authentic sample. Hence CYP199A2 catalyzed O-demethylation, most likely via hydroxylation of the methyl group to form the hemiacetal which spontaneously broke down to give formaldehyde and 4-hydroxybenzoate [9].

Crystallization of CYP199A2

We have obtained crystals of CYP199A2 without a bound substrate. Crystals of CYP199A2 (Fig. 2) were obtained by the vapor diffusion method and diffracted to *ca.* 2.0 Å resolution on an in-house diffractometer. Data collection, structure refinement, and substrate soaking experiments are in progress.

Conclusions

We have expressed four of the seven P450 enzymes of the metabolically diverse bacterium *Rhodopseudomonas palustris*. Palustrisredoxin A, a 2-Fe–2S ferredoxin that is associated with CYP199A2, has also been expressed. Substituted benzene compounds are found to be substrates

for three of the expressed P450 enzymes (CYP195A2, CYP199A2, and CYP203A1) but no substrate has been found for the fourth (CYP153A5). Monooxygenase activity was reconstituted for CYP199A2, but not CYP203A1 or CYP195A2, by using a Class I electron transfer chain comprising of putidaredoxin reductase from *Pseudomonas putida* and palustrisredoxin A. Crystals of the substrate-free CYP199A2 enzyme which diffract to ~2.0 Å have been obtained. The three-dimensional structure will provide valuable information on the important features of this new P450 enzyme. CYP203A1 and CYP195A2 must have alternative electron transfer proteins which may be found with further analysis of the *R. palustris* genome. Investigations of all these electron transfer proteins and their cross-reactivity to other P450 systems should provide valuable insights into electron transfer in P450 catalysis.

Acknowledgments

ZR is supported by the following grants from the National Science Foundation of China: Project "973" No. G1999075602 and Project "863" No. 2001AA233011. L.L.W thanks the Higher Education Funding Council for England for support, and the Engineering and Physical Sciences Research Council, UK, for a studentship to DC. We thank Neil Oldham for assistance with mass spectrometry experiments.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.01.133.

References

[1] S. Dagley, Catabolism of aromatic compounds by microorganisms, Adv. Microb. Physiol. 6 (1971) 1–46.
[2] C.S. Harwood, J. Gibson, Anaerobic and aerobic metabolism of diverse aromatic compounds by the photosynthetic bacterium *Rhodopseudomonas palustris*, Appl. Environ. Microbiol. 54 (1988) 712–717.
[3] F. Giffhorn, A. Kuhn, Phototrophic growth on citrate and regulations of citrate lyase in three strains of *Rhodopseudomonas palustris*, FEMS Microbiol. Lett. 7 (1980) 225–228.
[4] N. Elangovan, P.S.S. Gandhi, T. Kumaran, P.T. Kalaichelvan, Degradation of aromatic substances by a marine photosynthetic bacterium *Rhodopseudomonas palustris* (KK1), Ind. J. Environ. Protect. 20 (2000) 366–371.
[5] F.W. Larimer, P. Chain, L. Hauser, J. Lamerdin, S. Malfatti, L. Do, M.L. Land, D.A. Pelletier, J.T. Beatty, A.S. Lang, F.R. Tabita, J.L. Gibson, T.E. Hanson, C. Bobst, J.L. Torres y Torres, C. Peres, F.H. Harrison, J. Gibson, C.S. Harwood, Complete genome sequence of the metabolically versatile photosynthetic bacterium *Rhodopseudomonas palustris*, Nat. Biotechnol. 22 (2004) 55–61.
[6] S.G. Sligar, Nature's universal oxygenases: the cytochromes P450, Essays Biochem. 34 (1999) 71–83.
[7] I.G. Denisov, T.M. Makris, S.G. Sligar, I. Schlichting, Structure and chemistry of cytochrome P 450, Chem. Rev. 105 (2005) 2253–2277.
[8] F.P. Guengerich, The chemistry of cytochrome P450 reactions, Cytochromes P450: Metab. Toxicol. Aspects (1996) 55–74.
[9] F.P. Guengerich, Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity, Chem. Res. Toxicol. 14 (2001) 611–650.
[10] M.J. Cryle, J.E. Stok, J.J. De Voss, Reactions catalyzed by bacterial cytochromes P 450, Aust. J. Chem. 56 (2003) 749–762.
[11] C. Berne, B. Allainmat, D. Garcia, Tributyl phosphate degradation by *Rhodopseudomonas palustris* and other photosynthetic bacteria, Biotechnol. Lett. 27 (2005) 561–566.
[12] A.W. Munro, D.G. Leys, K.J. McLean, K.R. Marshall, T.W. Ost, S. Daff, C.S. Miles, S.K. Chapman, D.A. Lysek, C.C. Moser, C.C. Page, P.L. Dutton, P450 BM3: the very model of a modern flavocytochrome, Trends Biochem. Sci. 27 (2002) 250–257.
[13] R. De Mot, A.H. Parret, A novel class of self-sufficient cytochrome P450 monooxygenases in prokaryotes, Trends Microbiol. 10 (2002) 502–508.
[14] G.A. Roberts, A. Celik, D.J. Hunter, T.W. Ost, J.H. White, S.K. Chapman, N.J. Turner, S.L. Flitsch, A self-sufficient cytochrome P450 with a primary structural organization that includes a flavin domain and a [2Fe–2S] redox center, J. Biol. Chem. 278 (2003) 48914–48920.
[15] E.J. Mueller, P.J. Loida, S.G. Sligar, Twenty-five years of P450cam research. Mechanistic insights into oxygenase catalysis, in: P.R. Ortiz de Montellano (Ed.), Cytochrome P450: Structure, Mechanism, and Biochemistry, Plenum Press, New York, 1995, pp. 83–124.
[16] J.A. Peterson, M.C. Lorence, B. Amarneh, Putidaredoxin reductase and putidaredoxin—cloning, sequence determination, and heterologous expression of the proteins, J. Biol. Chem. 265 (1990) 6066–6073.
[17] T. Maier, H.H. Forster, O. Asperger, U. Hahn, Molecular characterization of the 56-kDa CYP153 from *Acinetobacter* sp. EB104, Biochem. Biophys. Res. Commun. 286 (2001) 652–658.
[18] J.B. van Beilen, E.G. Funhoff, Expanding the alkane oxygenase toolbox: new enzymes and applications, Curr. Opin. Biotechnol. 16 (2005) 308–314.
[19] J.B. van Beilen, R. Holtackers, D. Luscher, U. Bauer, B. Witholt, W.A. Duetz, Biocatalytic production of perillyl alcohol from limonene by using a novel *Mycobacterium sp*. cytochrome P450 alkane hydroxylase expressed in *Pseudomonas putida*, Appl. Environ. Microbiol. 71 (2005) 1737–1744.
[20] M. Nodate, M. Kubota, N. Misawa, Functional expression system for cytochrome P450 genes using the reductase domain of self-sufficient P450RhF from *Rhodococcus sp*. NCIMB 9784, Appl. Microbiol. Biotechnol. (2005) 1–8.
[21] K. Dus, R. Goewert, C.C. Weaver, D. Carey, C.A. Appleby, P-450 hemeproteins of *Rhizobium japonicum*. Purification by affinity chromatography and relationship to P-450CAM and P-450LM-2, Biochem. Biophys. Res. Commun. 69 (1976) 437–445.
[22] C.A. Appleby, G.L. Turner, P.K. Macnicol, Involvement of oxyleghemoglobin and cytochrome P 450 in an efficient oxidative phosphorylation pathway which supports nitrogen fixation in *Rhizobium*, Biochim. Biophys. Acta 387 (1975) 461–474.
[23] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, second ed., Cold Spring Harbor Laboratory Press, New York, 1989.
[24] S.G. Bell, X. Chen, R.J. Sowden, F. Xu, J.N. Williams, L.L. Wong, Z. Rao, Molecular recognition in (+)-alpha-pinene oxidation by cytochrome P450cam, J. Am. Chem. Soc. 125 (2003) 705–714.
[25] E. Deprez, C. Di Primo, G.H. Hoa, P. Douzou, Effects of monovalent cations on cytochrome P-450 camphor. Evidence for preferential binding of potassium, FEBS Lett. 347 (1994) 207–210.
[26] A.C. Westlake, C.F. Harford-Cross, J. Donovan, L.L. Wong, Mutations of glutamate-84 at the putative potassium-binding site affect camphor binding and oxidation by cytochrome P450cam, Eur. J. Biochem. 265 (1999) 929–935.
[27] M.J. Honeychurch, H.A.O. Hill, L.L. Wong, The thermodynamics and kinetics of electron transfer in the cytochrome P450cam enzyme system, FEBS Lett. 451 (1999) 351–353.
[28] M.D. Davies, S.G. Sligar, Genetic-variants in the putidaredoxin cytochrome P450cam electron-transfer complex—Identification of the residue responsible for redox-state-dependent conformers, Biochemistry 31 (1992) 11383–11389.