A Self-sufficient Cytochrome P450 with a Primary Structural Organization That Includes a Flavin Domain and a [2Fe-2S] Redox Center*

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P450 RhF from Rhodococcus sp. NCIMB 9784 is the first example of a new class of cytochrome P450 in which electrons are supplied by a novel, FMN- and Fe/S-containing, reductase partner in a fused arrangement. We have previously cloned the gene encoding the enzyme and shown it to comprise an N-terminal P450 domain fused to a reductase domain that displays similarity to the phthalate family of oxygenase reductase proteins. A reductase of this type had never previously been reported to interact with a cytochrome P450. In this report we describe the purification and partial characterization of P450 RhF. We show that the enzyme is self-sufficient in catalyzing the O-dealkylation of 7-ethoxycoumarin. The P450 RhF catalyzed O-dealkylation of 7-ethoxycoumarin is inhibited by several compounds that are known inhibitors of cytochrome P450. Pre-steady state kinetic analysis indicates that P450 RhF shows a 500-fold preference for NADPH over NADH in terms of $K_{p}$ value (6.6 μM versus 3.7 mM, respectively). Potentiometric studies show reduction potentials of −243 mV for the two-electron reduction of the FMN and −423 mV for the heme (in the absence of substrate).

Cytochrome P450 is a superfamily of monooxygenase enzymes with diverse catalytic activities. These have been found in all three phylogenetic domains of life, including many higher animal tissues. The primary chemical reaction catalyzed by these monooxygenases is the two-electron activation of molecular dioxygen, whereby one oxygen atom is inserted into the substrate with concomitant reduction of the second atom to water. NAD(P)H provides the required electron equivalents via a number of different reducter partners (1).

Depending on the nature of the reducter partner, P450 enzymes generally fall into two broad classes. Class I enzymes are three-component systems comprising an NAD(P)H-binding flavoprotein reductase, a small iron-sulfur protein, and the P450. These enzymes are found in the mitochondridial membranes of eukaryotes and in most bacteria. Class II enzymes are two-component systems comprising an FAD- and FMN-containing NADPH reductase (in which FAD and FMN are in an equimolar ratio) and the P450. This type of P450 is found almost exclusively in eukaryotes in association with the endoplasmic reticulum, where they play a major role in the oxidative metabolism of a wide spectrum of substrates including xenobiotics, endogenous fatty acids, steroids, leukotrienes, prostaglandins, and vitamins.

There are, however, examples of cytochrome P450 that cannot readily be defined as a member of either of these two classes. P450 BM3 (CYP102A1) from Bacillus megaterium, which oxidizes long chain fatty acids, comprises a diflavin reductase fused to the P450 to form a catalytically self-sufficient single polypeptide enzyme (2, 3). P450 BM3 has been likened to a fused eukaryotic class II enzyme. However, since its primary structural organization is quite distinct from the class II enzymes, P450 BM3 is regarded as the first example of a new, self-sufficient, class of P450 enzyme. Two further examples of P450 enzymes of this type (CYP102A2 and CYP102A3) from Bacillus subtilis were identified as a result of a whole genome sequencing project (4). A BM3-like P450 from the actinomycete Actinoaxynema pretiosum, which is thought to be involved in the biosynthesis of the polyketide antitumor agent ansamitocin, has also been described (5). Two membrane-bound eukaryotic counterparts of P450 BM3 have been cloned from Fusarium oxysporum (6) and Fusarium verticilloides (7).

Recently, a search against a data base of unfinished genome sequencing projects identified three more examples from Bacillus anthracis (Ames strain), Bacillus cereus, and the β-proteobacterium Ralstonia metallidurans (8).

During a PCR-based screen for new P450 activities from actinomycetes, we cloned a novel P450 gene from Rhodococcus sp. NCIMB 9784 (previously classified as Corynebacterium sp. Strain T1), which was of unique primary structural organization (9). Surprisingly, an analysis of the gene revealed that it encoded a heme domain fused to a reductase displaying convincing similarity to the phthalate family of dioxygenase reductases. We named the enzyme P450 RhF to reflect both its origin and the fused nature of the gene product. Despite the fact that proteins homologous to the C-terminal reductase portion of P450 RhF have been found in a diverse range of organisms, this was the first time an oxidoreductase of this type had been implicated in partnering a P450 enzyme. The N-terminal heme portion of the enzyme displays striking similarity to the thioacarbate-inducible P450 from Rhodococcus erythropolis NIs621 (ThcB), a class I enzyme of the CYP116 family (10). Working independently, De Mot and Parret (8) identified four putative homologues to P450 RhF from three pathogenic Burkholderia species and the heavy metal-resistant bacterium R. metallidurans by analyzing unfinished genomes with the ThcB sequence.
We have previously shown that a recombinant strain of *Escherichia coli* expressing the gene for P450 RhF was able to dealkylate 7-ethoxycoumarin, whereas the same strain lacking this gene was unable to do so. However, in these whole cell studies we could not definitively demonstrate that the enzyme was catalytically self-sufficient due to the possible involvement of endogenous electron transfer proteins. In this report we describe the isolation and partial characterization of recombinant P450 RhF.

**EXPERIMENTAL PROCEDURES**

**Enzymes and Chemicals**—All chemicals were purchased from Sigma-Aldrich. Biotinylated thrombin was supplied by Novagen (Madison, WI). A protease inhibitor mixture containing 4-(2-aminoethyl)benzenesulfonyl fluoride, benzamidine, pepstatin A, trans-epoxysuccinyl-l-leucylamido(4-guanido)butane, and N-ethylmaleimide(3-mercapto-3-thiopropanoyl)-Leu-Trp-(phosphoramidon) was supplied by Sigma-Aldrich. Restriction endonucleases, T4 DNA ligase, Vent DNA polymerase, and calf thymus alkaline phosphatase were from New England Biolabs (Beverly, MA).

**Maintenance and Growth of Microorganisms**—*Rhodococcus* sp. NCIMB 9784 was obtained from the National Culture of Industrial and Marine Bacteria (Aberdeen, UK). The strain was maintained on nutrient agar slopes at room temperature, and grown in Luria-Bertani (LB) medium (11) at 30 °C in baffled flasks. *E. coli* XL2-Blue MRF ultra competent cells were purchased from Stratagene (La Jolla, CA) and grown in LB medium at 37 °C. *E. coli* DH10B(DE3) cells were obtained from Invitrogen (Groningen, The Netherlands) and grown at 30 °C in LB medium unless stated otherwise. Ampicillin was used at 100 μg/ml when required for selection of plasmid on both liquid and solid medium. pET14b used in the expression studies was obtained from Novagen.

**DNA Manipulations**—Standard DNA procedures were used throughout (11). Total DNA was prepared from *Rhodococcus* sp. NCIMB 9784 as described previously (12). The full-length gene encoding P450 RhF was cloned into pET14b for expression to give a fusion protein with a 20-residue N-terminal prepeptide, which includes a His$_6$ tag and a thrombin cleavage site. The P450 RhF gene was amplified by PCR and cloned into the expression vector. The forward and reverse primers for negative control (11) were used to assess the banding pattern of expression. Protein was assessed by a standard procedure (11).

**Total Genomic DNA Preparation**—Total genomic DNA as template in a reaction volume of 50 μl contained 50 ng of *Rhodococcus* sp. NCIMB 9784 genomic DNA. The mixture was incubated at 95 °C for 2 min. The PCR mix included Vent DNA polymerase (New England Biolabs, MA), 10 mM MgCl$_2$, 200 μM of each deoxyribonucleotide triphosphate, 40 μM of each primer, 10% (v/v) dimethyl sulfoxide, and ~50 ng of *Rhodococcus* sp. NCIMB 9784 total genomic DNA as template in a reaction volume of 50 μl. The amplified nucleic acids were isolated and subjected with NdeI and BclI restriction endonucleases. The digested DNA was purified and cloned into the NdeI and BamHI sites of the pET14b vector to give the final expression construct (pAG04). Plasmid construction was performed in *E. coli* XL1-Blue MRF. The insert DNA was sequenced to ensure no mistakes had been introduced during the amplification process. pAG04 was then transformed into the expression strain *E. coli* BL21(DE3).

**Production of His$_6$-P450 RhF**—*E. coli* BL21(DE3) containing the expression construct was grown in LB medium containing 100 μg/ml ampicillin at 30 °C. We had previously demonstrated that a non-tagged version of P450 RhF was synthesized in a largely insoluble form when the culture temperature was elevated to 37 °C (9). After induction with 1 mM IPTG at an optical density (OD$_{600}$) of 0.8, growth was continued for up to 4 h before harvesting. Aliquots were withdrawn at regular time points. Expression was assessed by comparing the banding pattern obtained by SDS-PAGE analysis of whole cell extracts with that of a negative control (i.e. *E. coli* BL21(DE3) transformed with pET14b). The solubility of the protein was assessed by a standard procedure (11).

**Biochemical Characterization of P450 RhF**—The homogeneity of the protein preparation was assessed by SDS-PAGE using a 10–20% SDS-PAGE gel and electroblotted onto a nitrocellulose membrane (Hybond ECL, Amersham Biosciences) using a Bio-Rad transblot cell at a constant current of 150 mA for 90 min. After blocking in 5% (w/v) dried milk powder, the blot was subjected to immunodetection using mouse mono- clonal antibodies to 4-methylpyridine and dodecylphosphate conjugate (Sigma, Munich) in 0.1% trifluoroacetic acid to 0.05 g/ml and mixed with an equal volume of matrix (saturated solution of sinalpinic acid in 50% acetonitrile, 0.1% trifluoroacetic acid) on a stainless steel surface. The samples were air dried at room temperature to crystallize. The machine was operated in positive ion mode and calibrated with calbaminium and bovine serum albumin.

**Western Blotting**—Protein samples were resolved on 10–20% SDS-PAGE gels and electroblotted onto a nitrocellulose membrane (Hybond ECL, Amersham Biosciences) using a Bio-Rad transblot cell at a constant current of 150 mA for 90 min. After blocking in 5% (w/v) dried milk powder, the blot was subjected to immunodetection using mouse monoclonal antibodies to 4-methylpyridine and dodecylphosphate conjugate (Sigma, Munich) diluted 1:1000. Following extensive washing the blot was developed using 3,3′-diaminobenzidine in urea/hydrogen peroxide (FAST DAB tablets, Sigma-Aldrich) according to the manufacturers' guidelines.

**Spectroscopic Analysis**—The homogeneity of the protein preparation was assessed by SDS-PAGE using a 10–20% gradient gel. The spectral properties (ratio of P450-specific absorption at 420 nm compared with protein-specific absorption at 280 nm) were also measured. The cytochrome P450 concentration was determined by the method described in the literature (13), using an extinction coefficient of 14000 cm$^{-1}$ mol$^{-1}$ 1 cm$^{-1}$.

1 The abbreviations used are: IPTG, isopropyl-β-D-thiogalactopyranoside; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; OTTLE, optically transparent thin layer electrochemical.

2 Preliminary runs indicated that a recombinant strain of *Rhodococcus* sp. NCIMB 9784 was unable to dealkylate 7-ethoxycoumarin, whereas the same strain lacking this gene was unable to do so. However, in these whole cell studies we could not definitively demonstrate that the enzyme was catalytically self-sufficient due to the possible involvement of endogenous electron transfer proteins. In this report we describe the isolation and partial characterization of recombinant P450 RhF.
Results

Recombinant Expression—The expression construct (pAG04) was introduced into E. coli BL21(DE3), and the resultant cell extracts analyzed by SDS-PAGE. A band corresponding to the predicted molecular mass of the recombinant protein was visible on Coomassie-stained gels of the expression strain, which was absent from the control strain (E. coli BL21(DE3)pET14b). The identity of this band was confirmed by Western blot analysis using an anti-His tag antibody. Although we observed significant levels of recombinant protein from cell extracts of the expression strain grown in the absence of IPTG (i.e. leaky expression), protein purified from these preparations was generally subject to substantial proteolytic degradation. Subsequent analysis of whole cell extracts by Western blotting confirmed that much of this proteolysis was occurring in the cell prior to disruption (data not shown). In order to minimize this problem, all subsequent protein preparations were derived from cells grown in a fermentor to a low cell density (OD ~ 0.4), to induce with IPTG, and then harvested shortly afterward (see “Experimental Procedures”). The level of recombinant P450 RhF peaked at about 3 h after induction (Fig. 1).

His6-P450 RhF was purified using metal ion affinity and anion-exchange chromatography (Fig. 2). The His tag was then efficiently removed by treatment with thrombin. Removal of the tag was confirmed by both Western blot analysis using an anti-His tag antibody and by mass spectrometry (Fig. 3). We could detect no evidence of adventitious proteolysis at sites outside the targeted scissile bond.

Spectrophotometric Characterization—UV-visible absorption spectroscopy provides the basic technique for the recognition and characterization of cytochrome P450 enzymes. Recombinant His6-P450 RhF and recombinant P450 RhF lacking the His tag gave essentially identical UV-visible absorption spectra that are typical of P450 hemoproteins (14). The oxidized form of purified P450 RhF displays the general spectral properties of...
cytochrome P450 enzymes with the major Soret band located at 424 nm, and the smaller α and β peaks at 574 and 539 nm, respectively (Fig. 4). Upon reduction with sodium dithionite, the Soret band shifts to 423 nm and diminishes in intensity. Subsequent bubbling of CO through the solution of dithionite-treated P450 RhF resulted in the characteristic shift of the Soret band to 450 nm. The CO-difference spectrum displays the prominent peak at 450 nm (Fig. 4, inset). The spectral characteristics of the enzyme remained unchanged after 3 months storage at 20 °C in 50% (v/v) glycerol. P450 RhF with and without the His tag display the same spectral properties, suggesting that the presence of these additional residues at the N terminus does not perturb the heme environment. Interestingly when the His tag was engineered onto the C terminus of P450 RhF the purified enzyme displayed the same spectral characteristics but was enzymatically inactive (data not shown). In this case the His tag may have perturbed the [2Fe-2S] cluster, thereby interfering with the electron transfer process.

**Nucleotide Preference of P450 RhF**—In order to determine the pyridine-nucleotide preference of P450 RhF, both pre-steady state and steady state experiments were performed. The direct reduction of the FMN group was monitored using stopped-flow spectrophotometry and the resulting plots of $k_{\text{obs}}$ versus nucleotide concentration can be seen in Fig. 5. These gave rise to values for NADPH of $K_d = 6.6 \pm 0.8 \mu M$ and $k_{\text{lim}} = 180 \pm 5 \text{ s}^{-1}$ with the corresponding values for NADH being $K_d = 3.7 \pm 0.3 \text{ mM}$ and $k_{\text{lim}} = 111 \pm 5 \text{ s}^{-1}$. The P450 RhF catalyzed steady state oxidation of NAD(P)H using ferricyanide as an artificial electron acceptor also showed this cofactor preference. The NADPH-dependent reduction of potassium ferriyanide gave a $k_{\text{cat}}$ of $39 \pm 1 \text{ s}^{-1}$ and a $K_m$ of $6.6 \pm 0.4 \mu M$ whereas NADH gave a $k_{\text{cat}}$ of $16 \pm 2 \text{ s}^{-1}$ and a $K_m$ of $0.18 \pm 0.04 \text{ mM}$. These results make it clear that P450 RhF has a large preference for NADPH rather than NADH.

**Kinetics of 7-Ethoxycoumarin Dealkylation**—P450 RhF catalyzes the O-dealkylation of 7-ethoxycoumarin to form 7-hydroxycoumarin. The kinetic results obtained for His$_6$-P450 RhF were essentially the same as for the recombinant protein in which the His tag had been removed. The steady state kinetics of the O-dealkylation of 7-ethoxycoumarin obeyed Michaelis-Menten kinetics and a typical plot of rate versus substrate concentration is shown in Fig. 6. Steady state parameters, $k_{\text{cat}}$...
the fitting of the data very straightforward, giving reliable and accurate values. The two-electron reduction potential for the FMN group was found to be $-243 \pm 15 \text{ mV} (\text{versus NHE})$. It was not possible to accurately determine the two one-electron potentials for the oxidized/semiquinone and semiquinone/reduced couples of the FMN although there was evidence for a small amount of semiquinone formation (seen as a long-wavelength, 600 nm, absorbance in Fig. 8) during the potentiometric experiment. The one-electron reduction potential of the heme group, in the absence of any substrate, was determined to be $-423 \pm 10 \text{ mV} (\text{versus NHE})$.

**DISCUSSION**

Previously we had reported the cloning of P450 RhF, an unusual cytochrome P450 in which the heme domain is fused to a phthalate-family oxygenase reductase (9). The cloning strategy involved a PCR-based screen for P450-like sequence elements and required no prior knowledge of the target enzyme substrate specificity. Hence nothing is known about the likely natural function of P450 RhF, although we are currently investigating its substrate specificity by screening against a library of compounds.2 Nevertheless we reported that a recombinant strain of *E. coli* harboring the gene encoding P450 RhF is able to mediate the O-dealkylation of 7-ethoxycoumarin in whole cell biotransformation (9). However, the anticipated self-sufficiency of P450 RhF was not definitively demonstrated in these experiments since the possibility that endogenous *E. coli* redox proteins had supplied reducing equivalents to the recombinant protein in vivo could not be eliminated.

In the present study we have purified the recombinant Histagged P450 RhF to apparent homogeneity. The catalysis of the O-dealkylation reaction followed Michaelis-Menten kinetics and the steady state parameters for the reaction were determined. Subsequent removal of the His tag by treatment with thrombin did not affect enzyme activity. We have therefore demonstrated the ability of the isolated enzyme to mediate catalysis in the absence of additional redox proteins. P450 RhF represents the first example of a self-sufficient cytochrome P450 that does not possess a primary structural organization akin to that of P450 BM3.

Three general inhibitors of P450 enzymes were found to inhibit the O-dealkylation reaction and the $K_i$ value for each was determined. The three inhibitors are pyridine and imidazole derivatives that are thought to simultaneously bind to the lipophilic regions of the protein as well as the heme iron in a reversible manner (14). A double-reciprocal plot of enzyme kinetics in the presence and absence of inhibitor clearly demonstrated competitive inhibition, which confirms that the O-dealkylation reaction is indeed catalyzed by P450 RhF at the heme active site.

Direct measurement of the reduction of the FMN group by pyridine nucleotides showed that the enzyme displays a marked preference for NADPH over NADH. This degree of preference is almost entirely at the level of cofactor binding since there is only a very small difference (less than 2-fold) in the rate constant for FMN reduction whereas there is a very large (500-fold) difference in the dissociation constants. From sequence alignments and by analogy with the phthalate family of oxygenase reductase enzymes we had originally predicted that P450 RhF would be an NADH-dependent enzyme. Although P450 RhF uses FMN, in all other P450s it is FAD that receives the reducing equivalents from the pyridine nucleotide. In the microsomal P450s and P450 BM3, FMN is used to shuttle electrons from the FAD to the heme. Potentiometric analysis of P450 RhF shows that the FMN has a two-electron

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2. A. Çelik, personal communication.
reduction potential of $-243 \pm 15$ mV. This is well poised for reduction by NADPH which has a reduction potential around $-320$ mV. A value of $-243$ mV is not untypical for flavin-containing reductases and lies between the two-electron reduction potentials of the FAD and FMN in P450 BM3 which are around $-300$ and $-200$ mV, respectively (15).

The reduction potential for the heme, in the absence of substrate, was found to be $-423 \pm 10$ mV. This is identical, within experimental error, to the substrate-free heme potential seen for P450 BM3, reported as $-427 \pm 4$ mV (16). In P450 BM3 (and many other P450s) it has been shown that the binding of substrate causes a change in the spin state of the heme-iron, from low to high spin. Accompanying this spin state change is

**FIG. 6.** Michaelis-Menten curve for the NADPH-dependent $O$-dealkylation of 7-ethoxycoumarin catalyzed by P450 RhF. NADPH was used as the electron donor.

**FIG. 7.** Inhibitor studies of P450 RhF. Michaelis-Menten curves (top) and the corresponding Lineweaver-Burk plots (bottom) of the kinetics of $O$-dealkylation of 7-ethoxycoumarin in the absence (*) or presence of the inhibitor 1-phenylimidazole (○, 5 mM and ■, 10 mM).

**FIG. 8.** Potentiometric titration of intact P450 RhF. The upper panel shows visible absorption spectra of the key intermediate redox states of the protein during the titration: hemeox:FMNox (thick solid line), hemeox:FMNsq (thin solid line); hemeox:FMNred (thin hatched line); hemered:FMNred (thick hatched line). Lower panel shows the variation in absorbance with applied potential. Open circles correspond to absorption changes primarily associated with the heme ($A_{418 \text{ nm}}$, right axis), open-dot circles correspond to absorption changes primarily associated with the FMN ($A_{462 \text{ nm}}$, left axis). The data are fitted simultaneously to two-electron Nernst equations as described under “Experimental Procedures,” using Microcal Origin 7.
a large positive shift in reduction potential of around 130 mV (16). This change is spin-state and potential is required for efficient delivery of electrons from the reductase to the heme. Clearly a similar change would be required for efficient electron transfer to the heme in P450 RhF. Unfortunately, to date, binding trials with a variety of possible substrates have failed to produce any significant perturbation of the spin state. This lack of spin state change is probably the primary reason for the poor turnover rates and low coupling seen with a substrate such as 7-ethoxycoumarin.

Although the turnover of 7-ethoxycoumarin to 7-hydroxycoumarin is quite low (about 5 μmol of substrate turned over per 1 μmol of P450 RhF per minute), the primary objective of this study was to show self-sufficiency. Nevertheless, this single polypeptide electron transfer route could well support high catalytic activity. Indeed, the catalytic activity of P450 BM3 is the highest determined for a P450 monooxygenase (17,000 min⁻¹ for arachidonate) (17). Such high activity is due in large part to the highly efficient electron transfer from the NADPH cofactor via the reductase to the P450 heme within a single polypeptide chain (18, 19). The correlation between efficient electron transfer housed on a single polypeptide and a high level of substrate turnover, as observed for P450 BM3, is probably a general feature of this type of P450 enzyme.

The identity of the likely natural substrate(s) for P450 RhF is currently unknown. Discovery of a natural substrate will also help elucidate the physiological role of P450 RhF and possibly its counterparts in other organisms. As pointed out by De Mot and Parret (8) these enzymes are present in phylogenetically unrelated bacteria. It will be intriguing to determine whether the function of the enzymes is conserved among these diverse organisms.

In the well-characterized phthalate dioxygenase, the intramolecular electrons flow from NADH to the semiquinone FMN and then to the [2Fe-2S] redox cluster (20). The significant degree of sequence homology between these reductases and the C-terminal portion of P450 RhF suggests that they possess, mutatis mutandis, a similar mechanism. We are currently dissecting the individual electron transfer steps by a combination of stopped-flow studies involving intact P450 RhF and mixed domain fragments. Fundamental studies into the electron transfer mechanism will provide valuable information concerning domain interactions for efficient electron transport and catalysis in these enzymes.

Acknowledgments—We thank Hannah Florance (Institute of Cell and Molecular Biology, University of Edinburgh) for assistance with the MALDI-TOF analysis and Simon Daff (School of Chemistry, University of Edinburgh) for useful discussions.

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J. Biol. Chem. 2003, 278:48914-48920.
doi: 10.1074/jbc.M309630200 originally published online September 27, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M309630200

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