**Fusarium oxysporum** Fatty-acid Subterminal Hydroxylase (CYP505) Is a Membrane-bound Eukaryotic Counterpart of *Bacillus megaterium* Cytochrome P450BM3*

Received for publication, June 27, 2000, and in revised form, September 13, 2000
Published, JBC Papers in Press, September 19, 2000, DOI 10.1074/jbc.M005617200

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The gene of a fatty-acid hydroxylase of the fungus *Fusarium oxysporum* (P450foxy) was cloned and expressed in yeast. The putative primary structure revealed the close relationship of P450foxy to the bacterial cytochrome P450BM3, a fused protein of cytochrome P450 and its reductase from *Bacillus megaterium*. The amino acid sequence identities of the P450 and P450 reductase domains of P450foxy were highest (40.6 and 35.3%, respectively) to the corresponding domains of P450BM3. Recombinant P450foxy expressed in yeast was catalytically and spectrally indistinguishable from the native protein, except most of the recombinant P450foxy was recovered in the soluble fraction of the yeast cells, in marked contrast to native P450foxy, which was exclusively recovered in the membrane fraction of the fungal cells. This difference implies that a post-translation mechanism functions in the fungal cells to target and bind the protein to the membrane. These results provide conclusive evidence that P450foxy is the eukaryotic counterpart of bacterial P450BM3, which evokes interest in the evolutionary aspects concerning the P450 superfamily along with its reducing systems. P450foxy was classified in the new family, CYP505.

Cytochromes P450 are widespread in nature and catalyze monooxygenation along with a variety of other reactions (1–4). Despite the incomparable molecular diversity of the P450 superfamily, the tertiary structure is considered to be well conserved among all members (5, 6). This hypothesis is now being proven by an increasing body of knowledge of their crystal structures (7–12). The species P450nor and P450foxy were originally isolated from the fungus *Fusarium oxysporum* (13, 14). Both are unique in that they are self-sufficient, which means that they can complete their functions without the aid of other proteinaceous components such as NADPH-cytochrome P450 oxidoreductase (referred to as P450 reductase). P450nor functions as nitric-oxide reductase, taking part in the process of fungal denitrification. Its self-sufficient nature depends on its ability to receive electrons directly from NAD(P)H (13). In contrast to the unique reaction of P450nor, P450foxy catalyzes the subterminal (ω-1 to ω-3) hydroxylation of fatty acids (14) that seems to be a standard P450-dependent monooxygenase reaction, whereas it is also unusual because of its self-sufficient nature. P450foxy was suggested to be a fused protein of P450 and its reductase; and thus, the electrons from NADPH should transfer to the active-site heme via its putative reductase domain (14).

Fatty acid (or alkane) terminal (ω) and subterminal (in-chain; ω-1, ω-2, etc.) hydroxylations occur widely in nature and are catalyzed by multicomponent monooxygenase systems. The “terminal oxidases” of the bacterial systems except P450BM3 are of the non-heme iron type (15), whereas those of eukaryotes are of the P450 type (1, 4, 16). The physiological significance of the terminal (ω) hydroxylation of alkanes in bacteria and yeast is to utilize alkanes as carbon and energy sources via the β-oxidation of fatty acids that are formed by the successive oxidation of alkanes (17). On the other hand, the physiological roles of the terminal hydroxylation of fatty acids including those of P450BM3 and P450foxy are essentially unknown. In-chain hydroxylated fatty acids are located in cutins that protect plants from microbial attack (18). A contribution of P450-dependent fatty acid (eicosanoids) hydroxylation to the control of the arachidonate cascade in mammals has been proposed (1, 19).

P450BM3 (CYP102) of the bacterium *Bacillus megaterium* was the first member of the superfamily that was found to be fused with its reductase (20). P450foxy bears close resemblance to P450BM3 in terms of self-sufficiency, molecular mass, high catalytic turnover, and other enzymatic properties (14), suggesting that P450foxy is also a fused protein. Furthermore, P450foxy reacts with polychloroanilines, and antibodies raised against P450BM3 (14), indicating that they are quite similar. Limiting proteolysis of P450foxy yielded two main polypeptides that were separable on SDS-PAGE. Their Mr values suggest that the two polypeptides are derived from the P450 and reductase domains, respectively. Only the polypeptide with the lower Mr that should have been derived from the P450 domain reacted with the polyclonal antibodies raised against P450BM3 (14). This indicated similarity between the P450 domains of P450foxy and P450BM3. On the other hand, the only difference between P450foxy and P450BM3 was in their apparent intracellular localization. P450foxy was purified from the membrane.
fraction after solubilization (14), whereas P450BM3 is a soluble protein.

Here, we cloned the P450foxy-encoding gene and expressed it in a heterologous yeast host-vector system. The results showed close relationships between not only the P450 domains, but also the reductase domains of P450foxy and P450BM3, generating questions about the molecular evolution of P450 and its reductase.

**EXPERIMENTAL PROCEDURES**

**Microorganism Strains, Culture, and Media—**The *F. oxysporum* MT-811 fungal strain containing P450foxy (21) was the source of DNA. *Escherichia coli* XL1-Blue MRA, Y1090, and MV1190 were the host cells for λEMBL3, for λgt11, and for the construction of plasmids, respectively. *Saccharomyces cerevisiae* InvSc1 (Invitrogen) and its transformants were cultured in 2% dextrose and 0.67% yeast nutrient base (Difco) supplemented with 60 μg/ml l-leucine, 20 μg/ml l-histidine, and 40 μg/ml l-tryptophan; YEPD medium (1% yeast extract, Difco, 2% peptone, and 2% dextrose); and YEPG medium (1% yeast extract, 2% peptone, and 2% galactose). Recombinant P450foxy was produced by cultivating yeast transformants in 50 ml of YEPD medium in a 500-ml Erlenmeyer flask at 30 °C on a rotary shaker at 200 rpm. The cells were transferred 24 h later to YEPG medium and cultivated for >24 h under the same conditions.

**Peptide Sequencing—**Purified P450foxy (150 μg) (14) was resolved by SDS-PAGE, electroblotted onto a nitrocellulose membrane, and digested with trypsin as described (22). After separation by reverse-phase liquid chromatography, the amino acid sequences of the tryptic peptides obtained were determined using a PerkinElmer Life Sciences Procise 492 automated protein sequencer.

**Isolation of the P450foxy-encoding Gene (CYP505)—**Total DNA of *F. oxysporum* MT-811 (21) was the template for the polymerase chain reaction (PCR) with the following degenerate oligonucleotide primers: FA, 5'-TTAAGCCTGAGGAYGAGTA-3' (corresponding to FTA-FEDE); FB, 5'-CCRTGNC/CC/GTNGGNCRTC-3' (corresponding to DAE-AATG); FC, 5'-XAYGARCCNAAAYTGGGG-3' (corresponding to DEPNWG); and FD, 5'-ATRAAYTTTRCNGCRTT-3' (corresponding to NDADKF). The first PCR used 200 μM FA and 200 μM FB as the primers and 0.5 μg of total DNA as the template. Amplification proceeded by denaturation at 94 °C for 10 min, followed by 30 cycles of 94 °C for 0.5 min, 42 °C for 1 min, and 72 °C for 2 min and extension at 72 °C for 10 min. The second PCR used the product of the first PCR as the template and FC and FD as the primers under the same conditions.

Total DNA of *F. oxysporum* MT-811 was partially digested with Sau3AI, ligated to BamHi-digested λEMBL3, packaged in vitro, and transfected into *E. coli* XL1-Blue MRA. The resultant DNA library was screened by plaque hybridization (23) using the PCR product obtained above (see Fig. 1A) as the probe. Nucleotides were sequenced by dyeoxy chain termination (24) using the LCA dNTPs (Amersham Model 4200) automated DNA sequencer (LI-COR, Inc., Lincoln, NE). Southern Blot Analysis—Southern blot analysis essentially proceeded as described (23) using a nylon membrane (Hybond-N, Amersham Pharmacia Biotech). Probes were labeled and hybridized; membranes were washed; and signals were detected using the ECL nucleotide labeling and detection system (Amersham Pharmacia Biotech).

**Isolation of P450foxy cDNA—**A cDNA library of *F. oxysporum* MT-811 (25) was screened by plaque hybridization using the 1.4-kilobase pair of CYP505 (see Fig. 1A) as the probe. About 60% of the total cDNA that contained the 3'-end was obtained by this procedure. The 5'-region of the CYP505 cDNA was obtained by the rapid amplification of cDNA ends (RACE) as follows: mRNA was purified from total RNA of *F. oxysporum* (21) using an mRNA purification kit (Amersham Pharmacia Biotech). cDNA was synthesized using a Marathon kit (CLONTECH, Palo Alto, CA). The 5'-end of the cDNA was ligated to a 44-base adapter oligonucleotide (supplied with the kit) that included annealing sites for primers AP1 (5'-CACCCTGATACAGCCTCATTAGGGGC-3') and AP2 (5'-GACTCATATAGGCGCCTGAGCAGGC-3'). PCR was performed with the CYP505-specific primer 5'-2' (5'-TGTATTGGTTGATCCCAAGGCTAGTT-3') and primer AP1. The product was ligated as the template for the second PCR using the nested primer 5'-1' (5'-AGGATATTGATCTGGATATGACTGTGATGTC-3') and primer AP2. The product was subcloned into the pGEM-3Z vector (Promega, Madison, WI) and sequenced. The transcription initiation site was estimated from the results of 5'-RACE using the Marathon kit and primer R1(5'-CTGGGAATTCCTCATTAGGCTA-3').

**Expression in Yeast and Purification of Recombinant P450foxy—**The expression plasmid pYESfoxy was constructed as follows. The product of reverse transcription PCR was used as the template for PCR with the oligonucleotide primer YE1 (5'-GGGGTACCATGGCTGAATCTGTCC-3') and primer 5' - 1. The resulting PCR product was cloned into pGEM-T, the Km1-BamHI fragment of which was ligated to the same restriction sites of pYES2 (Invitrogen) to generate pYESfoxy. The ActII-SphI fragment of the CYP505 cDNA was inserted between the ActII and SpIi sites of pYESfoxy to generate pYESfoxy.

**S. cerevisiae** InvSc1 was transformed with pYES2 or pYESfoxy using lithium acetate (26). Induced cells were harvested by centrifugation and suspended in 10 ml of 50 mM MES (pH 7.0) containing 10% glycerol, 0.1 mM EDTA, and 1 mM dithiothreitol. The cells were disrupted twice using a French pressure cell press (30,000 p.s.i.) and then separated by centrifugation at 3000 × g for 10 min. The supernatant was fractionated by centrifugation at 150,000 × g for 80 min and used as the soluble fraction. The precipitate was solubilized with 0.15% Emulgen 913 (Rao Co., Tokyo) and separated by centrifugation at 150,000 × g for 80 min. The supernatant was used as the membrane fraction. Recombinant P450foxy was purified over DEAE-cellulose (DE52, Whatman) as described (14).

**Preparation of F. oxysporum Cellular Fractions—**Myelia of *F. oxysporum* MT-811 were collected on filter paper and disrupted as described (14). The cell-free extracts were fractionated by centrifugation at 150,000 × g for 90 min into supernatant (soluble fraction) and precipitate (membrane fraction). The membrane fraction was further solubilized in 0.5 M KCl and 0.1 M EDTA. The fraction was again separated by centrifugation at 150,000 × g for 90 min to obtain the solubilized fraction (supernatant).

**Western Blot Analysis—**Samples (10 μg of protein) were resolved by SDS-PAGE and electroblotted onto nitrocellulose membranes (Hybond-C, Amersham Pharmacia Biotech). The blot was analyzed using 2000-fold diluted anti-P450foxy antiserum raised against purified recombinant P450foxy. Immunoreactive signals were detected by chemiluminescence using the ECL detection system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

**Analytical Methods—**Absorption spectra were recorded using a Beckman DU7500 spectrophotometer. Fatty-acid hydroxylase and NADPH-cytochrome c reductase activities were measured as described (14). P450 content was determined using an extinction coefficient of 91 mM cm⁻¹ for the difference in absorbance between 450 and 490 nm (27). Protein content was determined using the Bio-Rad protein assay reagent. The amino acid sequences of proteins were aligned using GENETYX-MAC Version 10.1.1. A phylogenetic tree was constructed using the ClustalW Version 1.5 multiple alignment program (European Molecular Biology Laboratory).

**RESULTS**

**Isolation of the P450foxy-encoding Gene (CYP505)—**Based on the amino acid sequences of the tryptic peptides, four degenerate oligonucleotide primers (FA, FB, FC, and FD) were designed for nested PCR (28) against total DNA of *F. oxysporum* (Fig. 1A). A 920-base pair fragment with a deduced amino acid
sequence that contained portions of sequences exactly the same as those of the tryptic peptides (Fig. 2) was specifically amplified. This indicated that this fragment was derived from the gene for P450foxy. The genomic DNA library was subsequently screened using the 920-base pair fragment as a hybridization probe. One positive clone was selected and sequenced. An entire open reading frame was found (Fig. 2). The presence of five introns in the open reading frame was confirmed by comparison with the cDNA. The results of 5' RACE suggested that the transcription should start at position 2230 (Fig. 2). We de-

Fig. 2. Nucleotide and deduced amino acid sequences of CYP505. Introns are indicated in lowercase letters. The arrow indicates the predicted transcription initiation site. Putative CAAT boxes and poly(A)⁺ addition signal are underlined. Amino acid sequences of tryptic peptides are indicated by boldface underlining.
The P450 domain is composed of 1066 amino acid residues, and its calculated molecular mass is 117,871, which agreed well with that of purified P450foxy (118,000) estimated by SDS-PAGE (14). Hydrophobic clustering analysis suggested that the primary structure contained neither a membrane anchor sequence in its amino terminus nor a long hydrophobic stretch in the internal regions (data not shown), although P450foxy should be membrane-bound. The primary structure of P450foxy can be divided into P450, P450 reductase, and their linker domains from se-
catalytic function rather than the phylogenetic relationship of the organisms of their origins. The sequence identity of the P450 reductase domain of P450foxy was again the highest (35.3%) to the P450 reductase domain of P450BM3 (Table I) and much lower (<25%) to other P450 reductases. The extent of the identity was similar (24.9%) to the reductase of S. cerevisiae, the most closely related organism to F. oxysporum listed in Table I. Therefore, the sequence identities of both the P450 and P450 reductase domains of P450foxy are exceptionally high to the respective domains of P450BM3, although the reductase domains of P450foxy did not seem to react with the polyclonal antibodies to P450BM3 on Western blots (14). The sequence identity between the overall regions of P450foxy and P450BM3 is 37.3%. These results indicate that P450foxy and P450BM3 originated from the same ancient gene.

**Table I**

| Identity | Human (CYP4A11) | Rat (CYP4A1) | Housefly (CYP6A1) | A. thaliana (CYP97B3) | S. cerevisiae (CYP56) | B. megaterium (CYP102) |
|----------|-----------------|--------------|-------------------|-----------------------|----------------------|------------------------|
| P450 (family) | 22.7           | 23.5         | 21.6              | 26.3                  | 23.6                 | 40.6                   |
| P450 reductase | 24.5           | 25.0         | 26.1              | 26.1/26.2             | 24.9                 | 35.3                   |

*Percent identical residues are defined as 100 × (number of matched sites)/(sum of numbers of matched, substituted, and unpaired sites).*
mined using the native protein (14) (Table II). Thus, the exceptionally high catalytic turnover of native P450foxy as compared with other P450 monooxygenases could be reproduced in the recombinant protein. Furthermore, rP450foxy also exhibited the phenomenon observed with native P450foxy (14) as well as P450BM3 (37); its NADPH-cytochrome c reductase activity was enhanced in the presence of the substrate to be hydroxylated (fatty acid) (Table II). These results unequivocally demonstrate that the rP450foxy expressed in the yeast system is spectrally and kinetically indistinguishable from the native protein.

**P450foxy Is a Membrane Protein—**Native P450foxy is assumed to be a membrane-bound protein, but results have not been conclusive (14). We examined this property by Western blotting using the anti-P450foxy antiserum. As shown in Fig. 7A, a specific signal with an *M* of 118,000 reacted to the antiserum in both the cell-free extracts before fractionation and the membrane fraction, but not in the soluble fraction of *F. oxysporum*. P450foxy was partially released into the soluble fraction by washing the membrane fraction with 0.5 M KCl and mostly solubilized by 0.15% Emulgen 913 (Fig. 7B). These results suggest that P450foxy is loosely bound to the membrane. An immunoreactive signal was undetectable in the soluble fraction of the yeast cells, in which most of the rP450foxy was recovered in the membrane fraction. The native protein was experimentally found only in eukaryotes. Therefore, the genes for the cytochromes P450, P450 reductase is usually found as a single molecular species, except in plants, which have two (43). P450 and P450 reductase domains of P450foxy and P450BM3 appear to have fused after the eukaryotic and prokaryotic cells that occurred as an evolutionary event. Comparison of the reductase domains of P450foxy and P450BM3 with more reductases will clarify the origin of the fused proteins, although both the P450 and P450 reductase domains of the fused proteins seem to have originated in eukaryotes. Whether or not *F. oxysporum* contains an “usual” P450 reductase that is not fused with P450 requires investigation. The occurrence of another P450 reductase species in *F. oxysporum* is possible because the fungus should contain other P450 species, *e.g.* at least CYP51, which is essential for the synthesis of steroids (40, 41).

In addition to the P450 domain, the sequence identity of the P450 reductase domain of P450foxy is also exceptionally high to the corresponding domain of P450BM3 among the reductases examined (Table I). In contrast to the vast molecular diversity of P450, P450 reductase is usually found as a single molecular species, except in plants, which have two (43). P450 reductase of this type containing both FMN and FAD is fundamentally found only in eukaryotes. Therefore, the genes for the P450 and reductase domains of P450foxy and P450BM3 appear to have fused after *F. oxysporum* and *B. megaterium* diverged. This suggests a horizontal transfer of the fused gene between the eukaryotic and prokaryotic cells that occurred as an evolutionary event. Comparison of the reductase domains of P450foxy and P450BM3 with more reductases will clarify the origin of the fused proteins, although both the P450 and P450 reductase domains of the fused proteins seem to have originated in eukaryotes. Whether or not *F. oxysporum* contains an “usual” P450 reductase that is not fused with P450 requires investigation. The occurrence of another P450 reductase species in *F. oxysporum* is possible because the fungus should contain other P450 species, *e.g.* at least CYP51, which is essential for the synthesis of steroids (40, 41).

We also expressed the P450foxy-encoding gene in the heterologous host *S. cerevisiae*. The recombinant protein was catalytically and spectrally indistinguishable from the native protein, except for the fact that it was recovered mostly in the soluble fraction of the yeast cells. The native protein was exclusively recovered from the membrane fraction of fungal cells. We cannot yet explain why a small portion (20%) of rP450foxy was recovered in the membrane fraction. The *M* values of the native and recombinant protein species recovered in the soluble and membrane fractions were identical according to SDS-PAGE (Figs. 6 and 7). This does not necessarily rule out the possibility that these protein species are not completely iden-

### Table II

**Catalytic activities of native and recombinant P450foxy**

|                | Native | Recombinant |
|----------------|--------|-------------|
| **Laurate hydroxylase** | Kₘ for NADH | Cytochrome c reductase | Cytochrome c reductase with 0.15 m lauric acid |
| nmol NADPH min⁻¹ | nmol⁻¹ P450 | nmol⁻¹ | nmol⁻¹ |
| rP450foxy     | 1210 ± 110 | 0.15 ± 0.05 | 890 ± 50 | 1590 ± 140 |
| P450foxy      | 1200      | 0.16        | 900      | 2000      |

* Data are from Nakayama et al. (14).

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**DISCUSSION**

In this study, we cloned the gene of P450foxy (CYP505) of *F. oxysporum* and expressed it in heterologous host yeast cells. The results provide conclusive evidence that P450foxy is a fused protein of P450 and its reductase and that it has a phylogenetically close relationship to P450BM3 of *B. megaterium* as predicted (14), although they originated in a eukaryote and in a prokaryote, respectively. Genomic analysis has recently revealed that *Bacillus subtilis* contains two genes encoding a protein that is closely related to P450BM3 (PIR accession numbers A69975 and D68979), suggesting a wide distribution of P450BM3 among the genera *Bacilli*. Our findings regarding P450foxy further demonstrate that this type of fused protein would occur more often in nature across phyla than has been thought.

The phylogenetic relationships between the members of the P450 superfamily approximately agree with those between the source organisms of each P450 (4, 16, 39). For example, prokaryotic cytochromes P450 are clustered into a big branch that is separate from eukaryotic groups, and most plant cytochromes P450 are also clustered. However, two exceptions are known. Prokaryotic P450BM3 is classified in the same eukaryotic group as the CYP4 and CYP52 families (4, 16), and P450nor of *F. oxysporum* (CYP55) is classified in the group of prokaryotic cytochromes P450 (16, 39). These relationships also appear in Fig. 5. P450 may have emerged at a very early stage, possibly before eukaryotic cells appeared (16, 40), and the cytochromes P450 associated with steroidogenesis have retained the oldest functions of P450 (40). The recent finding of CYP51 (P45014DM) in prokaryotes (41, 42) has afforded further evidence to support this hypothesis. The appearance of fatty-acid (alkane) (sub)terminal hydroxylases of prokaryotes are mostly dependent on non-heme iron proteins (15), with the exception of P450BM3. The finding of P450foxy in a eukaryote is therefore intriguing.

In addition to the P450 domain, the sequence identity of the P450 reductase domain of P450foxy is also exceptionally high to the corresponding domain of P450BM3 among the reductases examined (Table I). In contrast to the vast molecular diversity of P450, P450 reductase is usually found as a single molecular species, except in plants, which have two (43). P450 reductase of this type containing both FMN and FAD is fundamentally found only in eukaryotes. Therefore, the genes for the P450 and reductase domains of P450foxy and P450BM3 appear to have fused after *F. oxysporum* and *B. megaterium* diverged. This suggests a horizontal transfer of the fused gene between the eukaryotic and prokaryotic cells that occurred as an evolutionary event. Comparison of the reductase domains of P450foxy and P450BM3 with more reductases will clarify the origin of the fused proteins, although both the P450 and P450 reductase domains of the fused proteins seem to have originated in eukaryotes. Whether or not *F. oxysporum* contains an “usual” P450 reductase that is not fused with P450 requires investigation. The occurrence of another P450 reductase species in *F. oxysporum* is possible because the fungus should contain other P450 species, *e.g.* at least CYP51, which is essential for the synthesis of steroids (40, 41).

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tical. However, removing a few amino acid residues from the N or C terminus by post-translational proteolysis is unlikely to have rendered most of the rP450foxy soluble because a small portion of the polypeptide comprising only a few amino acid residues probably cannot anchor the polypeptide to a membrane.

The distribution of a small portion of rP450foxy to the membrane fraction might have arisen from a nonspecific interaction.

On the other hand, native P450foxy would be targeted and bound to the membrane by a post (or co)-translational mechanism that functions in the fungal cells, but does not work in the yeast cells, because it was exclusively recovered in the membrane fraction of the fungal cells. The protein may be modified by a hydrophobic moiety such as a fatty acid (44) or a prenyl group (38). Such a modification is consistent with our findings that native P450foxy was loosely bound to the membrane and that the purified protein was inert against Edman degradation by a hydrophobic moiety such as a fatty acid (44) or a prenyl

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