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Cytochrome P450foxy, a Catalytically Self-Sufficient Fatty Acid Hydroxylase of the Fungus *Fusarium oxysporum*[1]

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We have purified membrane-bound fatty acid (ω-1-ω-3) hydroxylase of the fungus *Fusarium oxysporum* MT-811 and found that the activity depends on a single polypeptide with an apparent M_r value of 118,000. The purified hydroxylase exhibited spectral characteristics of cytochrome P450 (P450), and could catalyze the hydroxylation without the aid of any other proteinaceous components, such as NADPH-P450 reductase. These properties of the fungal hydroxylase are the same as those of bacterial P450BM3 of *Bacillus megaterium*, a catalytically self-sufficient fused protein of P450 and its reductase. Other properties of the two enzymes, such as molecular weight, high catalytic turnover, and the regiospecificity of the hydroxylating position, were also almost identical. Further, the fungal hydroxylase reacted with the antibody to P450BM3. It was thus shown that the fungal fatty acid hydroxylase structurally and functionally bears a close resemblance to P450BM3, although it is membrane-bound, unlike the bacterial counterpart. On the other hand, a unique phenomenon was found with the fungal hydroxylase: its NADPH-cytochrome *c*- or NADPH-menadione reductase activity was enhanced enormously upon binding of its substrate (fatty acid). This appears to be the first instance in which the reactivity of P450 reductase against an artificial electron acceptor was enhanced by the binding of the substrate (to be hydroxylated) to P450. These results raise interesting questions about the molecular evolution of P450. Here we term the fungal hydroxylase cytochrome P450foxy.

Key words: cytochrome P450, fatty acid hydroxylase, fungus, fused protein, NADPH-cytochrome P450 reductase.

Cytochrome P450 (P450), a superfamily of hemeproteins, is widely found in nature from bacteria to mammals, and usually catalyzes monooxygenation of a wide variety of endogenous and exogenous compounds (*1*). Since the discovery of P450, mammalian hepatic P450s have been studied intensively because of the medical implications. Recently, however, attention has also been drawn to P450s of other origins, such as those of microorganisms, plants, vertebrates, and invertebrates, because of their wide variety of functions. Further, recent advances in molecular cloning have made it possible to obtain P450 genes of those organisms without first isolating their products (proteins).

Few P450s have been found in bacteria and yeasts, whereas many have been found in fungi (*2*). However, there have been only a few reports of successful isolation of fungal P450s (*3-5*). This might reflect the low content and labile nature of membrane-bound fungal P450s. We have isolated a fungal P450 from *Fusarium oxysporum* MT-811 (*3*). Purification of this P450 was rather easy because of its soluble nature and the large content. Later, the unique physiological function of this P450 was revealed (*6*). It is involved in fungal denitrification, and acts as nitric oxide reductase (P450nor). We also found in the cell-free extracts of the fungus a fatty acid hydroxylase activity that exhibited P450-dependent properties (*7*). We afterwards showed that the hydroxylase activity might depend on a membrane-bound P450 that is distinct from the soluble one (P450nor) (*8*).

Fatty acid hydroxylase is widespread in nature, being present in mammals (*9-12*), plants (*13-15*), yeasts (*16-18*), fungi (*7, 19*), and bacteria (*20*). Most of these activities have been shown to be dependent on P450. The fatty acid hydroxylase activity of *F. oxysporum* more closely resembles in enzymatic properties (*7*) P450BM3 of *Bacillus megaterium*, a catalytically self-sufficient, fused protein containing both P450 and reductase domains (*21*), than the other P450s described above. So it is of great interest to know whether or not the fungal hydroxylase is also a fused protein. We have now purified the fatty acid hydroxylase from *F. oxysporum* and have shown that it is also a fused protein of P450 and its reductase, resembling P450BM3 with respect to structure and function.

MATERIALS AND METHODS

Microorganism—*F. oxysporum* MT-811, which contains P450 (*3*) and subterminal (ω-1-ω-3) fatty acid hydroxylase (*7*) and was firstly identified as a fungal denitrifier (*22*), was used throughout this work.

Cell Culture—*F. oxysporum* was cultured as previously

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described (3) in a 5-liter Ehrlenmeyer flask containing 3 liters of medium that consisted of 0.16% $(NH_4)_2SO_4$, 0.02% $MgSO_4 \cdot 7H_2O$, 2 ppm $FeSO_4 \cdot 7H_2O$, 20 ppm $FeCl_3 \cdot 6H_2O$, 2 ppm $CoCl_2 \cdot 6H_2O$, 0.2% soybean flour, 3% (v/v) glycerol, and 10 mM potassium phosphate (pH 7.5). The cells were incubated on a rotary shaker (130 rpm) at 27°C for 4 days, and then harvested by filtration.

Preparation of Microsomal Fraction—All operations were carried out below 4°C or on ice. The cells were disrupted as reported (7) with some modification. About 1.5 kg (wet weight) of cells was disrupted by grinding with aluminum oxide in 50 mM 2-[2-morpholino]ethanesulfonic acid (MES) buffer (pH 6.5) that contained 20% glycerol, 0.2 M KCl, 0.75 M sorbitol, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), and protease inhibitors (leupeptin, phenylmethylsulfonyl fluoride, and tosylphenylalanyl chloromethyl ketone, 0.2 mM each). The homogenate was centrifuged at $700 \times g$ for 15 min, and the supernatant was further centrifuged at $150,000 \times g$ for 80 min. The $150,000 \times g$ pellet was suspended in 10 mM MES buffer (pH 6.5) containing 10% glycerol, 0.1 mM EDTA, 1 mM DTT, and the protease inhibitors. The suspension was used as the microsomal fraction for further purification.

Solubilization and Purification of Fatty Acid Hydroxylase—All procedures were performed below 4°C. Unless otherwise stated, MES buffers (pH 6.5) were used throughout purification steps and all buffers contained 10% glycerol, 0.1 mM EDTA, and 1 mM DTT. The microsomal fraction (5 mg protein/ml) was solubilized by stirring with 0.15% Emulgen 913 (Kao, Tokyo) in 10 mM MES buffer for 1 h. The fraction was then centrifuged at $150,000 \times g$ for 80 min, and the supernatant was applied to a DEAE-cellulose (DE52, Whatman, UK) column (2.5×10 cm) equilibrated with 10 mM MES buffer that contained 0.05% Emulgen 913. After application, the column was washed with the equilibrating buffer, and then with 50 mM MES buffer that contained 0.05% Emulgen 913 until no further protein was eluted. The column was then eluted with a linear gradient of 0 to 0.3 M KCl in 50 mM MES buffer that contained 0.05% Emulgen 913. Active fractions were collected and adsorbed on an Octyl Sepharose CL-4B (Pharmacia, Sweden) column (2.5×20 cm) equilibrated with detergent-free 50 mM MES buffer. The column was washed with the equilibrating buffer and eluted with 0.5% Lubrol-PX (obtained from Nacalai Tesque, Kyoto) in the same buffer. The hydroxylase fraction was then loaded on a 2′,5′-ADP Sepharose 4B (Pharmacia, Sweden) column (15×25 mm) equilibrated with 50 mM MES buffer that contained 1 μM FAD, 1 μM FMN, and 0.05% Lubrol-PX. Elution was performed according to Black *et al.* (23) with some modification. After application, the column was washed in a stepwise manner with the following buffers in order: the equilibrating buffer, 500 mM MES buffer that contained 1 μM FAD, 1 μM FMN, and 0.05% Lubrol-PX, and then the 500 mM MES buffer that contained 10 mM 2′-AMP. The column was then finally eluted with 3 mM NADPH in the 500 mM buffer. To remove NADPH and protein-free flavins, the final eluate was applied to a P-6 DG (Bio-Rad, USA) column (2.5×40 cm) equilibrated with 50 mM MES buffer that contained 0.05% Lubrol-PX.

Assay of Fatty Acid Hydroxylase—Fatty acid hydroxylase was assayed as reported (24) by measuring the rate of conversion of [1-^{14}C]lauric acid (Amersham, UK) to the products, or spectrophotometrically by measuring the decrease in absorbance at 340 nm due to the oxidation of NADPH. The assay mixture, with a final volume of 0.5 or 1.0 ml, consisted of 0.5 mM fatty acid, 1 μM FAD, 1 μM FMN, 125 μM NADPH, and the enzyme preparation in 100 mM potassium phosphate buffer (pH 6.5) that contained 10% glycerol and 0.1% Emulgen 913. For assay of the purified enzyme, the reaction mixture contained 0.2-0.45 μg of protein. The reaction was initiated by adding a concentrated NADPH solution. The radioactivity was measured with an automatic TLC linear analyzer, Trace-master 20 (Bertold).

Stoichiometric Analysis—The assay mixture, with a final volume of 1.0 ml, consisted of the same components as in the standard procedure described above except that the concentration of lauric acid was lowered to 125 μM. The reaction was initiated by adding NADPH, and the decrease in absorbance at 340 nm was measured. After 30 min the reaction was stopped by the addition of 50 μl of 50% H_2SO_4, and then the reaction mixture was extracted with $CHCl_3$/CH_3OH (2 : 1). The $CHCl_3$ phase was removed and the aqueous phase was further extracted sequentially with ether and hexane. The $CHCl_3$, ether and hexane phases were combined and evaporated. The residue was analyzed by TLC as described previously (24). The radioactivity was measured as described above.

Assay of NADPH-Cytochrome c/Menadione Reductase—NADPH-cytochrome *c* reductase was assayed as reported (24). The reaction mixture, with a final volume of 1.0 ml, contained 50 μM cytochrome *c*, 1 μM FAD, 1 μM FMN, 125 μM NADPH, and the enzyme preparation in 100 mM potassium phosphate buffer (pH 6.5) that contained 10% glycerol and 0.1% Emulgen 913. The reaction was initiated by adding a concentrated NADPH solution, and the increase in absorbance at 550 nm was measured. In the case of stoichiometric analysis the concentration of NADPH was lowered to 50 μM NADPH. Reduction of cytochrome *c* and NADPH oxidation were followed simultaneously by measuring the changes in absorbance at 550 and 340 nm alternately.

NADPH-menadione reductase activity was assayed in the same reaction mixture except that cytochrome *c* was replaced with 0.2 mM menadione and that the decrease in absorbance at 340 nm was recorded.

Other Analytical Procedures—A UV 2100 spectrophotometer (Shimadzu, Kyoto) was employed for all spectrophotometric measurements.

P450 concentration was estimated by the procedure of Omura and Sato (25) employing the extinction coefficient of 91 $mM^{-1} \cdot cm^{-1}$ for the difference in absorbance between 450 and 490 nm.

SDS-PAGE was carried out by the method of Laemmli (26) employing a 7.5% gel for separation. Protein bands were revealed by staining with Silver staining II kit Wako (Wako Pure Chemical, Osaka).

Antibodies to P450BM3 (21) were kindly provided by Dr. Armand J. Fulco (University of California, Los Angeles). Western blot analysis of P450 was performed utilizing antibodies to P450BM3 after transferring proteins electrophoretically from SDS-PAGE gel to a polyvinylidene difluoride membrane (Clear blot membrane-p, Atto, Tokyo), by the method of Towbin *et al.* (27). The incubation conditions used for Western blot of the fungal P450

were those of Wen and Fulco (*28*).

Protein was determined using an assay kit (Bio-Rad, USA) or by the method of Lowry *et al.* (*29*), employing BSA as a standard.

RESULTS

Conditions for Induction of Fatty Acid Hydroxylase—Fatty acid hydroxylases of *B. megaterium* (P450BM3) (*20*), Jerusalem artichoke tuber (*14*), wheat (*15*), and *F. oxysporum* (*7*) resemble each other with respect to the regiospecificity of hydroxylation. This means that they catalyze subterminal (in-chain) hydroxylation of fatty acid. It is intriguing that these hydroxylases, except that of *F. oxysporum*, have all been reported to be induced by phenobarbital, a typical inducer of mammalian hepatic P450s, although they can not catalyze oxygenation of the inducer (*14, 15, 30*). So we examined here the effects on the fatty acid hydroxylase activity in *F. oxysporum* of several substances that are typical inducers of hepatic P450s. They are four barbiturates (barbital, phenobarbital, pentobarbital, and secobarbital), 3-methylcholanthrene, and clofibrate, which is widely used as an inducer for the P450 IV family. However, they did not increase the level of hydroxylase activity in *F. oxysporum* (data not shown).

Purification of Fatty Acid Hydroxylase Activity—The results of purification are summarized in Table I. The solubilization could be achieved with a rather low concentration (0.15%) of Emulgen 913. It seems likely that the enzyme is loosely bound to membranes. Throughout all purification procedures the hydroxylase activity was always eluted as a single peak, suggesting that it is due to a single component. The activity was not eluted from the 2′,5′-ADP Sepharose 4B affinity column with a high ionic strength solution or with 10 mM 2′-AMP, in marked contrast to a mammalian NADPH-P450 reductase that was easily eluted with 2 mM 2′-AMP from the same resin (*31*). Such a tight binding to the affinity resin was also observed with P450BM3 (*23*). The enzyme was unstable in the presence of NADPH or $NADP^+$. So a rapid desalting treatment was required.

SDS-PAGE—Purified fatty acid hydroxylase afforded a single band on SDS-PAGE with an apparent M_r value of 118,000 (Fig. 1A), showing that the activity is due to a single polypeptide, as expected (*24*).

Spectral Properties—The absorption spectra of the purified fatty acid hydroxylase were typical of P450, as shown in Fig. 2, with Soret peaks at 414, 410, and 448 nm, respectively, for the ferric, ferrous, and ferrous-CO complex species of the P450. The absorbance around 450 nm due to flavins was not observed in the native ferric form of the enzyme. We previously showed, however, that both FAD and FMN are obligatory for restoration of the activity (*24*). It would appear that flavins are easily released from the protein portion and thus their contents in the purified

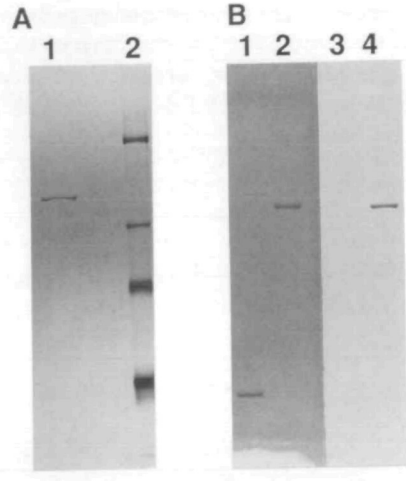

Fig 1 **SDS-PAGE and Western blotting of purified fatty acid hydroxylase.** A, SDS-PAGE. Lane 1, fatty acid hydroxylase, lane 2, standard proteins. Standard proteins are α_2-macroglobulin (M_r = 160,000), phosphorylase B (94,000), bovine serum albumin (67,000), and ovalbumin (43,000). B, Western blot analysis. Lanes 1 and 2, SDS-PAGE of purified P450nor and fatty acid hydroxylase, respectively, lanes 3 and 4, Western blotting of lanes 1 and 2, respectively. P450nor was purified as reported (*6*)

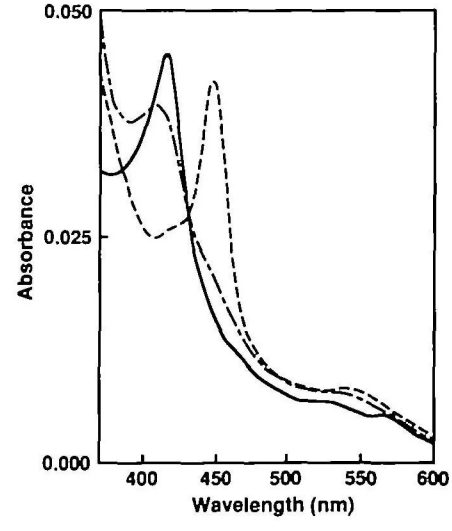

Fig. 2. **Absorption spectra of fatty acid hydroxylase.** —, ferric (native), – – –, ferrous (reduced with dithionite); ----, ferrous form complexed with CO, 90 μg protein in 50 mM MES buffer (pH 6.5) that contained 10% glycerol, 0 05% Lubrol-PX, 1 mM DTT, and 0.1 mM EDTA

TABLE I **Purification of fatty acid hydroxylase of *F. oxysporum*** Lauric acid was used as the substrate for the assay of the activity

Step	Protein (mg)	Specific activity (nmol/min/mg)	Total activity (μmol/min)	Recovery (%)	Specific P450 content (nmol/mg)
Microsome	13,300	2.4	32	100	nd[a]
Solubilized	4,000	5.0	20	63	nd
DEAE-cellulose	780	6 4	5.0	16	nd
Octyl Sepharose	270	30	8.1	25	nd
2′,5′-ADP Sepharose	0.34	6,500	2.2	6.9	3.0

[a]nd, not determined.

preparation are very low.

Optimal pH—The enzyme was most active at pH 6.5 at 25°C (data not shown). These conditions were employed for the standard assay.

Kinetic Constants—As reported previously, the fatty acid hydroxylase required either NADPH or NADH (*7*). The K_m for NADH was obtained as 0.16 mM from the steady-state kinetic analysis. However, the value of K_m for NADPH seemed to be too low to be determined, although the molecular activity (turnover number) was comparable for NADH (1,160 min^{-1}; apparent V_{max} value) and NADPH (1,200 min^{-1}; obtained under the standard assay conditions). At the NADPH concentration of 20 μM, the reaction was already saturated. So the K_m for NADPH seems to be of the order of 10^{-6} M or below. The expected K_m value is consistent with the tight binding of the enzyme to the affinity resin (2′,5′-ADP Sepharose 4B). The apparent K_m value for lauric acid was estimated as 150 μM. The turnover number (1,200 min^{-1} with respect to protein) seems to be comparable to that of P450BM3 (4,600 min^{-1} with respect to heme; Ref. *21*), when the low content of heme in the fungal preparation is taken into consideration, based on the low ratio of the absorbance at 414 and 280 nm (data not shown). Values of kinetic constants for the fungal hydroxylase were thus similar to those of P450BM3 (*21, 23, 32*), but quite different from those observed for other lauric acid hydroxylases (*14, 33*).

Substrate Specificity—The specificity with respect to the chain length of fatty acid was examined (Table II). The fungal hydroxylase exhibited the highest activity toward fatty acids with a chain length of C_{12}-C_{14}. The fungal enzyme is thus somewhat different in specificity from P450BM3, which exhibits the highest activity toward fatty acids with longer chain length (C_{15} and C_{16}).

P450BM3 can catalyze hydroxylation or epoxidation of fatty alcohols, fatty amides, and unsaturated fatty acids in addition to hydroxylation of saturated fatty acids (*21*). Reactions of the fungal P450 with these substances were also examined, as shown in Tables II and III. It seems that the fungal enzyme can also catalyze oxygenations of these substances, although the reactions were followed merely by measuring the decrease of NADPH spectrophotometrically. The fungal P450 was, however, almost inert towards alkanes.

Enhancement of the Electron Transfer from NADPH to Cytochrome c or Menadione by Substrates—We previously found that the NADPH-cytochrome *c* reductase activity of the fungal fatty acid hydroxylase is enormously enhanced by the presence of lauric acid, although this must be an artificial reaction (*24*). So we examined the effects of various substrates on the electron transfer from NADPH to an artificial electron acceptor, cytochrome *c* or menadione, catalyzed by the fungal hydroxylase. As shown in Table II, various fatty acids and related substances enhanced the NADPH oxidation (or cytochrome *c* reduction) in the presence of cytochrome *c* or menadione. The extent of enhancement by a substrate was essentially proportional to the hydroxylase activity of the enzyme towards the substrate itself. As indicated with lauric acid, hydroxylation of the substrate (fatty acid) occurred concomitantly with the reduction of cytochrome *c*, at a rate slightly lower than that in the absence of cytochrome *c*. This means that a portion of the electrons in FAD/FMN was utilized for the hydroxylation, although most of them were transferred to cytochrome *c*.

Stoichiometry—The stoichiometry of the enzymatic reactions was determined between NADPH oxidation, substrate (lauric acid) oxygenation, and/or cytochrome *c* reduction, as described in "MATERIALS AND METHODS." The stoichiometry between the NADPH oxidation and

TABLE II. **Substrate specificity and NADPH-cytochrome *c*/menadione reductase activity of the fungal fatty acid hydroxylase.** Each activity in the presence of the indicated substrate only, or both substrate and an artificial electron acceptor (cytochrome *c* or menadione), was determined spectrophotometrically by measuring the change in absorbance at 340 nm (substrate oxygenation and menadione reduction) or at 550 nm (cytochrome *c* reduction), as described in "MATERIALS AND METHODS."

Substrate	Substrate oxygenation[a] (nmol NADPH/min/mg)	Cytochrome *c* reduction[b] (nmol cyt. *c*/min/mg)	Menadione reduction[c] (nmol NADPH/min/mg)
No addition	421	4,260	4,520
Capric acid	3,670	20,100	18,900
Undecanoic acid	4,610		
Lauric acid	5,030 (100)[d]	38,000 (83)[d]	30,800
Tridecanoic acid	4,920		
Myristic acid	4,980	31,500	28,100
Pentadecanoic acid	4,191		
Palmitic acid	2,890	14,600	11,400
Heptadecanoic acid	2,380		
Stearic acid	1,641	11,900	8,210
Lauryl alcohol	932	10,700	9,050
Myristyl alcohol	721	6,330	4,690
Laurylamine	868	8,880	6,370
Myristylamine	762	7,140	5,970
Dodecane	609	4,410	
Tetradecane	527	4,490	

[a]Rate of NADPH oxidation in the presence of each substrate but absence of cytochrome *c*/menadione. [b]Rate of cytochrome *c* reduction in the presence of each substrate and cytochrome *c*. [c]Rate of NADPH oxidation in the presence of each substrate and menadione. [d]Relative activity of hydroxylation determined by using [1-^{14}C]lauric acid, as described in "MATERIALS AND METHODS."

TABLE III. **Substrate specificity of the fatty acid hydroxylase toward unsaturated fatty acids.** The activity was assayed as in Table II in the presence of each substance (0.5 mM). Each activity was compared with that for lauric acid (100%).

Substrate	Relative activity(%)
Palmitoleic acid	63.3
Oleic acid	50.0
Linoleic acid	63.5
α-Linolenic acid	28.4
γ-Linolenic acid	57.0

TABLE IV. **Stoichiometry between NADPH oxidation and cytochrome *c* reduction in the presence or absence of fatty acid (lauric acid) substrate for cytochrome *c* reductase activity of the fatty acid hydroxylase.** The method is indicated under "MATERIALS AND METHODS." Amounts of NADPH oxidized and cytochrome *c* reduced, during the first 3 min of the reaction, are indicated.

	Lauric acid only	Cytochrome *c* only	Lauric acid +cytochrome *c*
NADPH oxidized (nmol)	1.3	9.8	25.4
Cytochrome *c* reduced (nmol)	—	10.1	22.4

substrate oxygenation in the absence of cytochrome *c* was almost 1:1 (1:0.86), as expected (data not shown). As shown in Table IV, however, the stoichiometry between the NADPH oxidation and cytochrome *c* reduction in the absence of lauric acid was also 1:1. These stoichiometries seemed to be unchanged under conditions in which both reactions (substrate oxygenation and cytochrome *c* reduction) were occurring concomitantly, because the extent of NADPH oxidation in the presence of both lauric acid and cytochrome *c* was almost identical to the sum of the amount of cytochrome *c* reduced in the system and the amount of NADPH oxidized due to the substrate oxygenation alone (*i.e.*, in the absence of cytochrome *c*).

Western Blot Analysis—As shown in Fig. 1B, the purified fungal hydroxylase reacted with antibodies raised against P450BM3. By contrast, P450nor isolated from the same fungus did not react with them. The results imply that there are structural homologies between the fungal hydroxylase and P450BM3.

DISCUSSION

We have isolated two distinct P450 species from the fungus *F. oxysporum*, P450nor, nitric oxide reductase involved in the fungal denitrifying system (*6*), and the fatty acid hydroxylase (present study). This seems to be a rare instance of the molecular diversity of P450 found in a single species of microorganism. We term the newly isolated cytochrome P450foxy (derived from both fatty acid oxygenase and *F. oxysporum*). Its content in the microsomal fraction was extremely low, in spite of the distinct activity. Its successful isolation depended on the use of the affinity chromatography with 2′,5′-ADP Sepharose.

P450foxy resembles P450BM3 in many respects. They are catalytically self-sufficient, since they are fused proteins of P450 and its reductase. The absolute requirement of both FAD and FMN for the restoration of the activity (*24*) suggests that P450foxy contains a flavoprotein domain similar to NADPH-cytochrome P450 reductase, as P450BM3 does. P450foxy and P450BM3 also bear a close resemblance to each other in the regiospecificity of the hydroxylating position (ω-1-ω-3), catalytic turnover, high affinity for NADPH, and the M_r value, although their preferences in the chain length of substrates are somewhat different. Further, P450foxy reacted with the antibody to P450BM3. These results demonstrate that both fungal and bacterial P450s are very similar to each other not only in catalytic but also in structural properties, although P450foxy is membrane-bound, unlike P450BM3.

By contrast, enhancement of the electron transfer from NADPH to an artificial electron acceptor upon addition of a substrate is a phenomenon characteristic of P450foxy. It is generally observed that binding of the substrate to a P450 enhances reduction of the P450 itself by its reductase or ferredoxin (*34*). This phenomenon arises from changes caused by the binding of substrate in the heme-environment or in the heme itself, such as in the spin state (*34*). The enhancement of cytochrome *c* or menadione reductase activity observed with P450foxy means that the binding of a substrate to P450 affects the reactivities of not only P450, but also of its reductase. Further studies such as kinetic analyses are required to elucidate this unique phenomenon, along with the unusual stoichiometry between the NADPH oxidation and cytochrome *c* reduction (Table IV).

According to Gotoh (*35*) each P450 can be classified into either of two major classes, that is, B-class and E-class. B-class consists of bacterial P450s and E-class, of eukaryotic P450s. However, there are a few exceptions. Fungal P450nor (P450 55A) belongs to the B-class and bacterial P450BM3 (P450 102A) is grouped into the E-class. P450BM3 thus resembles mammalian P450s in the IV family. However, the present results suggest that P450BM3 is systematically related more closely to P450foxy than to any other P450 known to date. There has been no instance of "orthologous" (*36*) P450 gene products that are distributed among both eukaryotic and prokaryotic cells. Our results described here raise such a possibility.

In contrast to the close similarity between the proteins, P450foxy was not induced by barbiturate, unlike P450BM3. It is intriguing that expression of all barbiturate-inducible P450 genes of eukaryotes and prokaryotes seems to be regulated by the same mechanism (*37*). These facts indicate that the structural and regulatory portions of the genes for these P450s (P450BM3 and P450foxy) have diverged separately. *F. oxysporum* has been thus shown to contain unique P450s (P450foxy and P450nor) that raise intriguing questions concerning the molecular evolution of P450.

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