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FATTY ACID HYDROXYLASE OF THE FUNGUS *FUSARIUM OXYSPORUM* IS POSSIBLY A FUSED PROTEIN OF CYTOCHROME P-450 AND ITS REDUCTASE

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Summary: Fatty acid subterminal (ω-1~ω-3) hydroxylase of the fungus *Fusarium oxysporum* was solubilized from the microsomal fraction and partially purified. The hydroxylase activity was recovered into a single active fraction, and its spectral nature showed the presence of cytochrome P-450 (P-450). Fatty acid hydroxylase activity was markedly restored upon addition of FAD, FMN, and/or hemine to the eluted fraction. The fraction also exhibited other properties characteristic of both a hemeprotein and a flavin-containing reductase. These results are highly indicative that the fungal hydroxylase is a fused protein containing both P-450 and its reductase domains. In this aspect the fungal enzyme resembles bacterial P-450$_{BM3}$, although it is membrane-bound unlike the bacterial counterpart.

We have observed that *F. oxysporum* contains distinct species of P-450 in both soluble and microsomal fractions (1). The soluble one had been already isolated (2) and its physiological function was recently found as unique nitric oxide reductase involved in the fungal denitrification, and its trivial name was thus termed P-450nor (3). We suggested that fatty acid subterminal (ω-1, ω-2 and ω-3) hydroxylase activity detected in the cell-free extract (4) might dependent on the microsomal P-450 (1). Its catalytic properties resemble those of P-450$_{BM3}$ of *Bacillus megaterium*, a unique fused protein of P-450 and reductase domains (5).

There has been little paper that reports successful isolation of fungal P-450 from microsomal fractions although many fungi have been shown to contain P-450 (6-14). This might depend on the instability of fungal P-450s against solubilizing treatments. P-450nor is the exception since it is produced in a large amount and recovered in the soluble fraction (1-3). It is also noteworthy that multiple molecular species of P-450 with distinct functions have not been isolated from an individual microorganism. So far as known occurrence of P-450 among microorganisms, in particular among bacteria, is generally poor as compared with higher class organisms. Despite the difficulty we examined to isolate the microsomal P-450

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of *F. oxysporum* and to associate it with the hydroxylase activity. In this paper we report that the fungal fatty acid hydroxylase activity depended on a P-450, as was expected, that might be a fused protein of P-450_{BM3} type.

Methods

The microsomal fraction was prepared from the cells of *F. oxysporum* (500g wet cells) as reported (1). Fatty acid hydroxylase was assayed as reported (4) using [^{14}C]lauric acid as substrate, or spectrophotometrically by measuring the decrease in absorbance at 340 nm due to NADPH. The reaction mixture, with total volume of 1 ml, consisted of 0.5 mM lauric acid, 0.5 mM NADPH, and the enzyme solution in 20 mM potassium phosphate buffer (pH 7.0) containing 10% glycerol, and 0.1% Emulgen 913 (Kao). The complete system was further supplemented by 1 μM FAD and 1 μM FMN. NADPH-cytochrome *c* reductase was assayed by a modification of the method of (15). Fatty acid hydroxylase was solubilized from the microsomal fraction with 0.1% Emulgen 913 as described in the legend to Fig. 1. Then the solubilized fraction was applied to the affinity chromatography with 2',5'-ADP Sepharose 4B (Pharmacia). The column (15 × 25 mm) was equilibrated with 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol, 0.1 mM EDTA, and 0.1% Emulgen 913. The column was washed after application of the sample with the buffer, and then with the same buffer containing 5 mM AMP. The activity was not eluted by these washing treatments. The column was then eluted with the same buffer containing 1 mM NADPH. The activity was eluted as a single peak almost at the front with NADPH. The hydroxylase fraction was then applied to a Fast Desalting column (Pharmacia) equilibrated with 20 mM potassium phosphate buffer (pH 7.0) containing 10% glycerol, 1 mM dithiothreitol, 0.1 mM EDTA and 0.1% Lubrol-PX, equipped with an FPLC system (Pharmacia), to remove NADPH and to replace Emulgen 913 with Lubrol-PX.

Results and Discussion

Among various detergents tested Emulgen 913 was the best to solubilize the microsomal hydroxylase without loss of activity, as shown in Fig. 1. About 40% of the activity seemed to be solubilized by merely washing without detergent. In this case, however, a portion of the activity in the supernatant was filtrated with a micro-filter (pore size 0.2 μm), suggesting that the activity was not really solubilized. It would appear that some portion of membranes was disrupted into smaller pieces by the washing treatment and could not be precipitated by the ultra-centrifugation. Emulgen 913 with the concentration of only 0.1% was effective in solubilizing the activity. In this case the activity was not filtrated and further purification was possible as described below. So the activity was judged to have been solubilized.

Fig. 2 shows absorption spectra of the active fraction eluted from the affinity column. They are characteristic of a heme protein and the ferrous-CO (carbon monoxide) complex exhibited the Soret peak at 446 nm, showing that the fraction contained a P-450. Table I shows effects of several substances on the hydroxylase activity. Addition of FAD and FMN was necessary to reconstitute the activity (complete, in the table). Further addition of hemine restored the

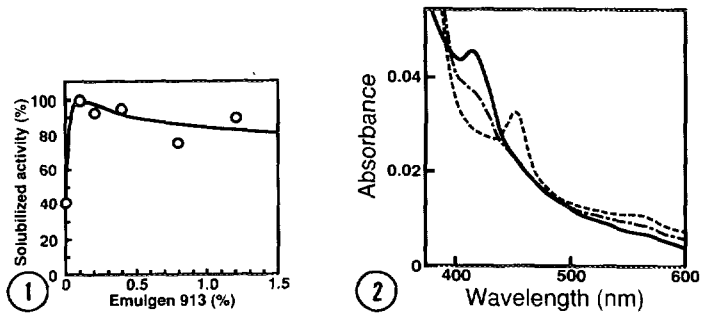

Fig. 1. Solubilization of fatty acid hydroxylase with Emulgen 913.
The microsomal suspension (5.4 mg protein/ml) was stirred in the presence of indicated concentration of Emulgen 913 at 4°C for 60 min, and then centrifuged at 100,000 × g for 60 min. The sum of hydroxylase activity recovered in the resulting supernatant was estimated as solubilized and represented as % of the total activity detected in the original microsomes.

Fig. 2. Absorbance spectra of fatty acid hydroxylase fraction.
——, native; — — —, reduced with dithionite; - - - -, bubbled with CO for 30 s and then reduced with dithionite.

activity enormously. The activity was inhibited by CO, cyanide, and menadione, whereas as observed previously (4), not inhibited by metyrapone. The affinity column is expected to bind NADPH-binding proteins, and thus to bind reductases but not P-450, however, the eluted active fraction contained P-450. And further, effects of inhibitors or activators (Table I) showed that the activity exhibits properties of both a flavoprotein and a hemeprotein. On considering together with its catalytic resemblance to P-450$_{BM3}$, it is highly possible that the fungal hydroxylase is also a fused protein consisting of P-450 and its reductase domains.

As shown in Fig. 3, the hydroxylase fraction revealed NADPH-cytochrome *c* reductase activity, one of properties characteristic of NADPH-P-450 reductase (16). It is of interest that the substrate of hydroxylase (lauric acid) enormously enhanced the reductase activity. The result is also highly indicative that both the reductase and hydroxylase are fused into a single protein and have intimate interactions with each other.

The absorption spectra of ferric, ferrus, and the CO-complex of the fungal hydroxylase (Fig. 2) also seem to resemble each corresponding spectrum of P-450$_{BM3}$, although a shoulder at 450-475 nm due to flavins was not obviously observed in the spectrum of the native, oxidized form of the fungal enzyme. The lack of the shoulder might depend on a low content of flavins in the enzyme preparation, which is supported by the enormous restoration of activity upon addition of FAD and FMN (Table I). It has been reported with the artificially fused protein of P-450 and NADPH-P-450 reductase that addition of a substrate enhanced the NADPH oxidase activity of the fused protein or the anaerobic reduction of heme and flavin chromophores with NADPH (17). The enhancement by lauric acid of cytochrome *c* reductase activity of the fungal enzyme (Fig. 3) along with these activations by substrate

Table I. Cofactors and inhibitors of fatty acid hydroxylase

Additives	% Activity
Complete	100
- FAD	0
- FMN	51
Hemine (1 mM)	239
KCN (1 mM)	0
CO : O_2 (80:20)	0
Metyrapone (1 mM)	104
Menadione (0.3 mM)	29
o-Phenanthroline (1 mM)	84
8-Hydroxyquinoline (1 mM)	102
Diethyldithiocarbamate (1 mM)	88

The hydroxylase activity was assayed as described in Methods. The complete system was supplemented with FAD and FMN. Indicated amount of each substance was further added to, or omitted from (-), the complete system.

observed with the artificially fused protein might reflect the general mechanism observed among P-450 systems that the binding of a substrate to P-450 enhances the electron flow from NADPH to P-450.

The protein content in microsomal fraction of the fungal fatty acid hydroxylase seems to be very low in spite of its distinct activity (Nakayama & Shoun, unpublished). This indicates

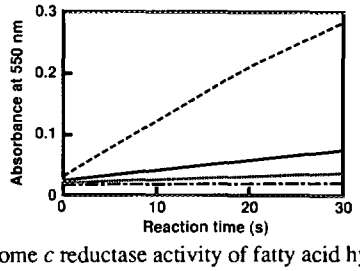

Fig. 3. NADPH-cytochrome *c* reductase activity of fatty acid hydroxylase fraction.

The reaction mixture, with the final volume of 1.0 ml, contained 40 μM cytochrome *c* (horse heart, Sigma), 100 μM NADPH, 1 μM FAD, 1 μM FMN, and the enzyme in 100 mM potassium phosphate buffer (pH 7.0) containing 10% glycerol and 0.1% Emulgen 913. For the line of experiment where lauric acid was added, its final concentration was 250 μM. The reaction was initiated by adding NADPH, and the increase in absorbance at 550 nm was recorded.

——, complete; — — —, minus hydroxylase fraction; -------, minus NADPH; - - - -, plus lauric acid.

that the enzyme exhibits a high catalytic turnover, like P-450$_{BM3}$. The previously observed spectrum that showed the presence of microsomal P-450 (1) might depend on another P-450 species. It is thus likely that *F. oxysporum* contains more than 2 molecular species of P-450.

The present results demonstrated that microsomal fatty acid hydroxylase of *F. oxysporum* depends on a P-450, as was expected. It resembles soluble P-450$_{BM3}$ of *B. megaterium* in several aspects, whereas activation of its cytochrome *c* reductase activity by fatty acid was not observed with P-450$_{BM3}$. Further, molecular multiplicity of P-450 in *F. oxysporum* has become more possible. Comparison of (deduced) amino acid sequences between the fungal hydroxylase and P-450$_{BM3}$ would be much interesting because they must differ in their intracellular localization. Although it is rather difficult to purify the fungal P-450 because of its low productivity, we are trying further purification.

References

1. Shoun, H., Sudo, Y., Seto, Y., and Beppu, T. (1983), *J. Biochem.* **94**,1219-1229.
2. Shoun, H., and Tanimoto, T. (1991), *J. Biol. Chem.* **266**, 11078-11082.
3. Nakahara, K., Tanimoto, T., Hatano, K., Usuda, K., and Shoun, H. (1993), *J. Biol. Chem.* **268**, 8350-8355.
4. Shoun, H., Sudo, Y., and Beppu, T. (1985), *J. Biochem.* **97**, 755-763.
5. Narhi, L. O., and Fulco, A. J. (1986), *J. Biol. Chem.* **261**, 7160-7169.
6. Ambike, A. H., Baxter, R. M., and Zahid, N. D. (1970), *Phytochemistry* **9**, 1953-1958.
7. Desjardins, A. E., and VanEtten, H. D. (1986), *Arch. Microbiol.* **144**,84-90.
8. Jermings,J. C., Coolbaugh, R. C., Nakata, D. A., and West, C. A. (1993), *Plant Physiol.* **101**,925-930.
9. Guan, J., and De Waard, M. A. (1992), *Pestic. Biochem. Physiol.* **43**,152-161.
10. Ballard, S. A., Kelly, S. L., Ellis, S. W., and Troke, P. F. (1990), *J. Med. Veter. Mycol.* **28**, 327-334.
11. Madyastha, K. M., Jaynathi, C. R., Madyastha, P., and Sumathi, D. (1984), *Can. J. Biochem. Cell Biol.* **62**, 100-107.
12. Cerniglia, C. E., and Gibson, D. T. (1978), *Arch. Biochem. Biophys.* **186**, 121-127.
13. Breskvar, K., Cresnar, B., Plaper, A., and Hudnik-Plevnik, T. (1991), *Biochem. Biophys. Res. Commun.* **178**, 1078-1083.
14. VAN Gorcom, R. F. M., Boschloo, J. G., Kuijvenhoven, A., Lange, J., VAN Vark, A. J., Bos, C. J., VAN Balken, J. A. M., Pouwels, P. H., and VAN den Hondel, C. A. M. J. J. (1990), *Mol. Gen. Genet.* **223**,192-197.
15. Dignam, J. D., and Strobel, H. W. (1977), *Biochemistry* **16**, 1116-1123.
16. Dignam, J. D., and Strobel, H. W. (1975), *Biochem. Biophys. Res. Commun.* **63**, 845-852.
17. Fisher, C. W., Shet, M. S., Caudle, D. L., Martin-Wixtrom, C. A., and Estabrook, R. W. (1992), *Proc. Natl. Acad. Sci. USA* **89**, 10817-10821.