# IDENTIFICATION OF THE ω-HYDROXYLASE OF *PSEUDOMONAS OLEOVORANS* AS A NONHEME IRON PROTEIN REQUIRING PHOSPHOLIPID FOR CATALYTIC ACTIVITY*

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Summary: Fatty acid and alkane hydroxylation by the inducible, cyanide-sensitive enzyme system of *Pseudomonas oleovorans* involves three enzymes: NADH-rubredoxin reductase, rubredoxin, and an ω-hydroxylase. The ω-hydroxylase is a yellow protein containing one atom of iron per polypeptide chain of molecular weight 42,000. Catalytic activity is lost upon removal of the iron and is restored when ferrous ions are incubated with the apohydroxylase. Hydroxylase preparations from which most of the phospholipid has been removed exhibit decreased activity unless supplemented with a phospholipid fraction from this bacterium or with dilauroylglyceryl-3-phosphorylcholine.

Three protein components, as well as NADH and molecular oxygen, are required for the hydroxylation of fatty acids and hydrocarbons by *Pseudomonas oleovorans* (1,2). These are rubredoxin, NADH-rubredoxin reductase, and the ω-hydroxylase. Rubredoxin, a nonheme iron protein with a molecular weight of 19,000, is capable of binding up to two atoms of iron per molecule (3-5) and catalyzes alkyl hydroperoxide reduction (6,7) as well as electron transfer for hydroxylation. The reductase, a flavoprotein with a molecular weight of 55,000, contains one molecule of FAD (8,9). The ω-hydroxylase, which is relatively unstable, was purified to a specific activity of 590 (nmoles of octane hydroxylated per min per mg of protein at 30°) and shown to occur as an aggregate of high molecular weight and to contain iron and traces of FAD and heme (10).

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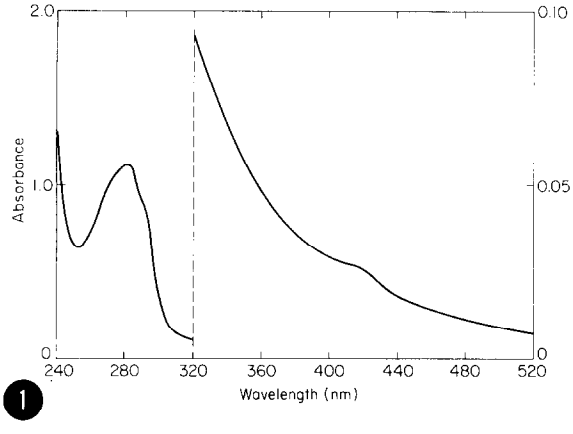


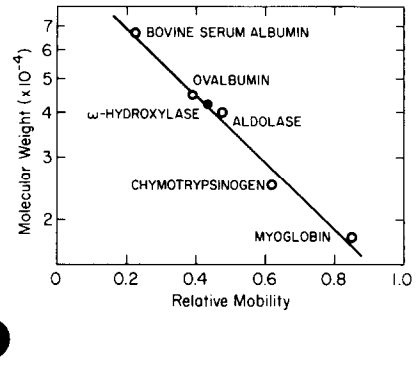


Fig. 1: Absorption spectrum of ω-hydroxylase (eluate from DEAE-cellulose; 0.4 mg of protein per ml) recorded in an Aminco DW-2 spectrophotometer in cuvettes of 1-cm light path.

Fig. 2: Estimation of molecular weight of ω-hydroxylase subunits by SDS-polyacrylamide gel electrophoresis. A mixture of the ω-hydroxylase (eluate from Agarose A-50m; 10 μg of protein) and the standard proteins (5 μg each) was treated with SDS and mercaptoethanol and submitted to electrophoresis in 10% polyacrylamide gel in the presence of SDS as described by Weber and Osborn (19).

The present studies indicate that the ω-hydroxylase contains at least one atom of iron per polypeptide chain, that the iron is required in the catalytic activity of this protein, and that phospholipid must be present for hydroxylation activity. This yellow enzyme is an unusual example of a mixed-function oxidase containing nonheme iron as the prosthetic group. Fisher et al. (11) have recently shown that rat liver phenylalanine hydroxylase is an iron enzyme, and several other reports have indicated a requirement of $Fe^{2+}$ for monooxygenase activity, as with the camphor ketolactonase of a pseudomonad (12), p-hydroxybenzoate hydroxylase (13), bovine adrenal tyrosine hydroxylase (14), and p-anisate O-demethylase of a pseudomonad (15,16).

Cultures of *P. oleovorans* grown on a medium containing hexane as the carbon source (10) were harvested, and the cells were stored as a frozen paste. The ω-hydroxylase was solubilized by sonication and purified by a modification of procedures described elsewhere (10) to yield enzyme preparations with specific activities as high as 2,000. Protein concentrations were estimated

according to Murphy and Kies (17). In the experiments to be described the various operations were carried out at 4° unless otherwise stated. Highly purified ferredoxin-NADPH reductase (18), generously provided by Dr. Graham Palmer, and NADPH were substituted for NADH-rubredoxin reductase and NADH in the hydroxylation assays.

The purified ω-hydroxylase is a yellow protein with the absorption spectrum shown in Fig. 1. The enzyme exhibits some absorbance in the visible region, but the only distinguishing characteristic is a slight shoulder at about 416 nm. In the ultraviolet region the maximum is at 283 nm. This spectrum is similar to that reported for rat liver phenylalanine hydroxylase (11). In ω-hydroxylase preparations dialyzed extensively against Tris-acetate buffer, iron was shown to be present, but no significant amount of copper, manganese, molybdenum, cobalt, chromium, or selenium could be detected by neutron activation in the Michigan Ford nuclear reactor, and no labile sulfide was found when the purified hydroxylase was analyzed by a slight modification of published methods.

The ω-hydroxylase, which is isolated as an aggregate,has an apparent molecular weight of $2 \times 10^6$ (10). When the purified preparation was treated with sodium dodecyl sulfate (SDS) and mercaptoethanol and submitted to electrophoresis in 10% polyacrylamide gel, a single major band accounting for at least 99% of the protein was observed, along with several very faint minor bands. As shown in Fig. 2, a comparison of the migration of the major band with that of standard proteins indicated a molecular weight of about 42,000. The iron content, expressed as atoms per polypeptide chain, is 1.0 to 1.2 in the best preparations.

Evidence that iron plays an important role in the function of the hydroxylase was obtained by the experiments presented in Table I. The addition of ferrous ions to the purified enzyme gave a small but significant stimulation, indicating the presence of small amounts of apoenzyme. Removal of most of the iron by chelation with EDTA after reduction by dithionite resulted in extensive

Table I

Removal of iron from ω-hydroxylase and reconstitution of activity

| Enzyme preparation | Iron content (nmoles per mg protein) | Specific activity |
|---|---|---|
| ω-Hydroxylase | 24 | 386 |
| ω-Hydroxylase + ferrous ions | | 520 |
| ω-Hydroxylase treated with EDTA to remove iron | 3.2 | 35 |
| Apohydroxylase + ferrous ions | | 350 |

The iron content of the hydroxylase preparations was determined by atomic absorption spectrometry. The octane hydroxylation activity was estimated at 30° by a radioactive assay as described previously (10). When the effect of added iron was tested, the holoenzyme or apoenzyme preparation was incubated in 0.05 M Tris buffer, pH 7.4, containing 0.1 mM $Fe(NH_4)_2(SO_4)_2$ for 10 min at room temperature; an aliquot was then removed for assay so that the final concentration of iron in the reaction mixture was 0.01 mM.

For removal of iron from the hydroxylase, 0.025 ml of 0.1 M EDTA and 0.025 ml of 0.1 M $Na_2S_2O_4$ were added to the enzyme solution (DEAE-cellulose eluate; 7.4 mg of protein in 2.0 ml of 0.05 M Tris buffer, pH 7.4), and the mixture was dialyzed about 18 hours each against 250 ml of 0.5 M Tris buffer, pH 7.4, containing 1.0 mM EDTA and 1.0 mM dithionite, against 500 ml of Tris buffer containing 1.0 mM EDTA, and against 500 ml of Tris buffer. This procedure was carried out at 4°, and nitrogen gas was bubbled through the solution during dialysis.

loss of activity, but this was largely restored when the iron-depleted preparation was incubated with ferrous ions prior to assay for octane hydroxylation activity. In other experiments it was found that the activity of the EDTA-treated enzyme was not restored by the addition of ferric ions.

Data indicating that phospholipid is required for maximal activity of the ω-hydroxylase are given in Table II. It is clear that upon removal of phospholipid from the enzyme by treatment with ammonium sulfate in the presence of cholate, a large loss of activity occurred. The addition of synthetically prepared dilauroylglyceryl-3-phosphorylcholine (dilauroyl-GPC) to such preparations largely restored the initial activity. In other experiments it was found that a phospholipid fraction obtained by chloroform-methanol extrac-

Table II

Effect of phospholipid on activity of ω-hydroxylase

| Preparation | Phospholipid content (nmoles per mg protein) | Specific activity | |
|---|---|---|---|
| | | Without added phospholipid | With added dilauroyl-GPC |
| ω-Hydroxylase (30-35% ammonium sulfate fraction) | 487 | 855 | 942 |
| Same after cholate treatment and precipitation by ammonium sulfate | 44 | 344 | 863 |
| Same after a second cholate treatment and precipitation by ammonium sulfate | 9 | 131 | 900 |

To decrease the phospholipid content, sodium cholate was added to a final concentration of 5 mg per ml in a hydroxylase preparation containing 1.0 mg of protein per ml (final concentration) in 0.05 M Tris buffer, pH 7.4, and solid ammonium sulfate was then added to 40% saturation. The mixture was stirred for 15 min and then centrifuged for 10 min at 25,000 X g, and the precipitate was taken up in 0.1% cholate in Tris buffer and again centrifuged. The slight precipitate was discarded, and the supernatant fraction was diluted in the same buffer-cholate mixture before use. The phospholipid content was determined by the method of King (20) after extraction according to Bligh and Dyer (21), and the catalytic activity was measured as the octane-dependent rate of NADPH oxidation (10).

tion of a hydroxylase preparation also stimulated the activity of the cholate-treated enzyme but was not as effective as dilauroyl-GPC. Deoxycholate failed to replace the phospholipid when tested at concentrations from 50 to 200 μg per ml in the hydroxylation assay system. The phospholipid requirement may account in part for the instability of the enzyme preparations during purification and storage.

The results presented indicate that nonheme iron is the prosthetic group of the *P. oleovorans* ω-hydroxylase. This enzyme catalyzes not only the hydroxylation of fatty acids and alkanes (10), but also, as shown more recently,

the epoxidation of alkenes (22). In contrast, a hemoprotein, cytochrome P-450, catalyzes the same reactions as well as acting on a variety of other substrates in liver microsomes (23), hydroxylates camphor in *Pseudomonas putida* (24), and has also been found in extracts of an octane-utilizing *Corynebacterium* (25).

A phospholipid requirement has also been established for the activity of cytochrome P-450 in the reconstituted liver microsomal enzyme system (26,27). The lipid facilitates electron transfer from NADPH to cytochrome P-450 (28) and apparently exerts its effect without causing the formation of aggregates or membrane-like structures (29). Whether phospholipid has a similar effect on electron transfer from reduced pyridine nucleotides to the ω-hydroxylase remains to be established.

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