Characterization of the ω-Hydroxylase of *Pseudomonas oleovorans* as a Nonheme Iron Protein[1]

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The ω-hydroxylase of *Pseudomonas oleovorans*, which catalyzes the hydroxylation of fatty acids and alkanes and the epoxidation of alkenes in the presence of a reduced pyridine nucleotide, a reductase, rubredoxin, and molecular oxygen, has been purified to electrophoretic homogeneity. Octane hydroxylation and octadiene epoxidation activities appear to remain at a constant ratio during the purification procedure. The hydroxylase has been characterized as a nonheme iron protein containing one iron atom and one cysteine residue per polypeptide chain of molecular weight 40,800. The enzyme is inhibited by cyanide, and activity is restored upon removal of the cyanide by dialysis. Iron is removed from the enzyme by dialysis against EDTA provided that a reducing agent such as dithionite or ascorbate is also added, and enzyme activity is restored by the addition of ferrous ions to the apohydroxylase.

As described previously, the hydroxylation of fatty acids and hydrocarbons by extracts of *Pseudomonas oleovorans* (1, 2) requires molecular oxygen and a reduced pyridine nucleotide and is catalyzed by an inducible multienzyme system composed of rubredoxin, NADH–rubredoxin reductase,[3] and the ω-hydroxylase[4] (3, 4). The rubredoxin has been purified to homogeneity and characterized as a red nonheme iron protein with a molecular weight of 19,000. It is capable of binding up to two atoms of iron per molecule (5, 6), and the epr[5] spectrum of the oxidized form is characteristic of high-spin ferric ions in a rhombic field (7, 8). The reductase, which has also been obtained in a homogeneous state, contains one molecule of FAD per polypeptide chain and has a molecular weight of 55,000 (9, 10). In contrast to rubredoxin and the reductase, the ω-hydroxylase is relatively insoluble, somewhat unstable, and forms aggregates with an apparent molecular weight of about 2×10^6 (11). Evidence has been presented in a brief report that the ω-hydroxylase requires phospholipid for full activity and contains about one atom of iron per polypeptide chain (12).

The present paper describes the properties of an electrophoretically homogeneous preparation of the ω-hydroxylase with a specific activity over three times as great as that described previously (11). This enzyme is an unusual example of a monooxygenase which contains nonheme iron as the prosthetic group and may have, at

[3] Systematic name, NADH:rubredoxin oxidoreductase, EC 1.6.7.2.

[4] Systematic name, alkane, reduced-rubredoxin:oxygen 1-oxidoreductase, EC 1.14.15.3.

[5] Abbreviations used: epr, electron paramagnetic resonance; DEAE, diethylaminoethyl; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); FAD, flavin–adenine dinucleotide.

most, one cysteine sulfur atom as a ligand bound to iron.

EXPERIMENTAL PROCEDURES

Purification of ω-hydroxylase. The hydroxylase was purified by the method of McKenna and Coon (11) but with the following modifications, which improved the overall yield more than twofold and the specific activity of the final preparation about threefold. Glycerol and EDTA were omitted from the buffers. The culture medium was initially at 23°C, and the temperature did not exceed 28°C during bacterial growth. The harvested cells, which are stored as a frozen paste, were lysed in 0.05 M Trischloride buffer, pH 7.4; after centrifugation, the supernatant fraction was discarded and the residue was suspended in buffer and adjusted to pH 7.4. The preparation was sonicated for 12 min while the temperature was maintained at 6°C in an ice–salt bath, the pH was adjusted to 7.4, and streptomycin sulfate was then added. The resulting extract had a specific activity of 0.10 in a typical preparation. The fraction precipitated by 30 to 35% ammonium sulfate (specific activity, 0.73; yield, 66%) was centrifuged at 100,000g, and the supernatant solution (11 ml; 700 mg of protein) was submitted to descending column chromatography on an agarose (Bio-Gel A-50m) column (3.5 × 54 cm) previously equilibrated with 0.05 M Tris buffer, pH 7.4, containing no KCl. The column was eluted under hydrostatic pressure with the same buffer at a flow rate of about 25 ml/h, and 12-ml fractions were collected. Two major protein peaks were eluted, the second of which contained the ω-hydroxylase. Enzyme of highest activity usually appeared in tubes 15 to 25 (specific activity, 2.1; overall yield from the starting bacterial extract, 31%). The preparation was then submitted to column chromatography on DEAE-cellulose equilibrated with 0.05 M Tris buffer, pH 7.4. The column was washed with the same buffer and eluted with a similar buffer solution containing 0.15 M KCl. The enzyme was eluted in a single protein peak with a specific activity of 2.0, representing an overall yield of 17% from the starting extract. Although this step does not usually increase the specific activity significantly, it removes nucleic acids (11) and traces of other contaminants. The enzyme preparation was concentrated by ultrafiltration in an Amicon Diaflo cell (Model 202) using an XM-50 membrane and stored at −70°C at a protein concentration of 3 to 6 mg/ml. The final preparations are faintly yellow in color and show a trace of turbidity. The enzyme is stable to storage under these conditions for at least several months but is stable at 4°C for only a few days. Unless indicated otherwise, purified ω-hydroxylase eluted from DEAE-cellulose was used in the experiments to be described.

For preparation of the apoenzyme, the ω-hydroxylase (10 mg of protein in 2 ml of 0.05 M Tris buffer, pH 7.4) was dialyzed for 8 h at 4°C against 2 liters of 0.2 M Tris buffer, pH 7.4, containing 15 mM EDTA and 10 mM dithionite and then overnight at the same temperature against 2 liters of buffer alone. The buffers were flushed with nitrogen gas prior to use, and the flasks were sealed during dialysis. The resulting preparations were about 90% apoenzyme. Repetition of the dialysis procedure increased the apoenzyme content to 95% but was not carried out routinely because with such preparations the addition of iron resulted in less recovery of holoenzyme. In some instances the apoenzyme was extracted with 1:2 chloroform–methanol according to the method of Bligh and Dyer (13) to prepare the delipidized protein.

Other methods. The ω-hydroxylase was assayed at all stages of purification by the spectrophotometric method described previously (11), which measures the octane-dependent oxidation of NADPH in a reaction mixture containing spinach ferredoxin-NADP reductase and bacterial rubredoxin as electron carriers and the ω-hydroxylase as the rate-limiting component. One unit of ω-hydroxylase is defined as the amount catalyzing the hydroxylation of 1.0 μmol of octane per minute at 30°C, and the specific activity is expressed as units per milligram of protein. Protein concentrations were determined by the method of Murphy and Kies (14) using crystalline bovine serum albumin as standard.

For amino acid analysis, enzyme samples which had been extensively dialyzed against 50 mM phosphate were hydrolyzed in 5.7 N HCl in sealed tubes under nitrogen at 110°C for various time intervals. After removal of the hydrochloric acid by repeated concentration under reduced pressure, the amino acids were determined with a Beckman 120C amino acid analyzer (15). The values are expressed relative to the alanine content. Tryptophan was determined after hydrolysis with mercaptoethane sulfonic acid according to Penke *et al.* (16) and half-cystine was determined after performate oxidation. Polyacrylamide gel electrophoresis was carried out at 10°C in the presence of sodium dodecyl sulfate in a discontinuous buffer system according to Laemmli (17); the exact conditions used for denaturing the protein samples and staining the gels are given elsewhere (18). Metals were determined with a Perkin-Elmer Model 306 atomic absorption spectrophotometer; reagent-grade iron wire and copper wire dissolved in nitric acid served as standards.

Materials. Electrophoretically pure rubredoxin (A_{280}:A_{497} ratio = 7.9) was isolated from *P. oleovorans* by a method described previously (5), and highly purified ferredoxin–NADP reductase from spinach was generously provided by Dr. Martha L. Ludwig. NADPH, *o*-phenanthroline, DTNB, and DEAE-cellulose (medium mesh; 1.0 mequiv/mg) were ob-

tained from Sigma. The DEAE-cellulose was thoroughly washed with acid and alkali before use as described earlier (11). Agarose (Bio-Gel A-50m, 50 to 100 mesh) was obtained from Bio-Rad Laboratories, urea and guanidine of highest purity were from Schwarz/Mann, sodium dodecyl sulfate was from BDH Chemicals, n-octane (research grade, for use as substrate) and n-hexane (technical grade, for use in the bacterial growth medium) were from Phillips Petroleum, and 1,7-octadiene was from Columbia Carbon Co., Princeton, New Jersey.

RESULTS

Gel electrophoresis. Preparations of the ω-hydroxylase purified as described under Experimental Procedures were submitted to sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis; the results are shown in Fig. 1. The enzyme exhibited a single band and was estimated to have a molecular weight of 40,800 by comparison with standard proteins. Similar results were obtained when the apoenzyme or delipidized apoenzyme was submitted to gel electrophoresis. In earlier experiments some enzyme preparations exhibited a doublet in the 40,000 molecular weight region, with a molecular weight difference of about 1000. This was probably due to contamination of the bacterial strain, since cultures grown from a single cell exhibited a single band corresponding to the lower band of the doublet. In the absence of a detergent the ω-hydroxylase is faintly turbid and forms high molecular weight aggregates (11).

Analysis of purified hydroxylase for various components. Some analytical data on samples of the most purified ω-hydroxylase preparations are given in Table I. Iron was present in the amount of one atom per polypeptide chain, whereas copper, heme, flavin, and acid-labile sulfide were present at barely detectable levels. The isolated hydroxylase has a high content of phospholipid, binding, on the average, 20 molecules per polypeptide chain. This observation and the need for sonication to remove the enzyme from the bacteria suggest that it may be membrane-bound in the cell; van Eyk and Bartels (23) have come to a similar conclusion concerning the alkane hydroxylase of *P. aeruginosa.* Rubredoxin and NADH–rubredoxin reductase, both of
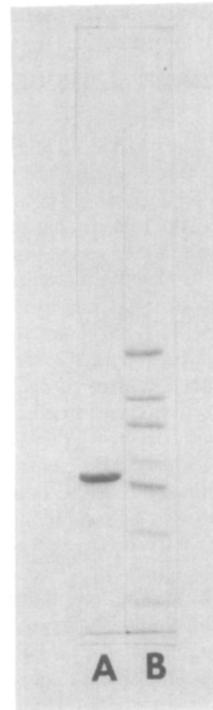

FIG. 1. Sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis of ω-hydroxylase preparations in a discontinuous buffer system. Following treatment with sodium dodecyl sulfate and mercaptoethanol, the ω-hydroxylase and standard proteins (chymotrypsinogen, lactate dehydrogenase, aldolase, ovalbumin, glutamate dehydrogenase, catalase, and bovine serum albumin) were submitted to electrophoresis. Migration was from top to bottom; the location of the front was determined with a tracking dye. A, ω-hydroxylase (1 μg); B, standard proteins (0.4 μg each).

which have distinct bands in the ultraviolet region, could not be detected by examination of the spectrum of the ω-hydroxylase (12) or by enzymatic assay. Since these two proteins have a much lower molecular weight than the ω-hydroxylase, they are undoubtedly removed during purification of the enzyme by agarose gel chromatography.

The purified hydroxylase has a faint yellow color which is more pronounced at protein concentrations as high as 30 to 40 mg/ml. When lipids were extracted from the protein with 1:2 chloroform–methanol, it was apparent that the pigment was present in the extract. On silicic acid column

Component	Concentration (nmol/nmol of polypeptide)
Iron	0.98
Copper	0.03
Heme	<0.01
Flavin	<0.01
Acid-labile sulfide	0.07
Phospholipid	21

[a] Iron and copper were determined by atomic absorption, heme was determined by the pyridine hemochrome method (19), flavin was determined as described previously (11), acid-labile sulfide was quantitated by a modification (20) of the method of Fogo and Popowsky (21), and phospholipids were determined by extraction of the protein according to Bligh and Dyer (13) followed by determination of the phosphorus content of the extract according to King (22). The amount of polypeptide was calculated on the basis of protein content and the known monomeric molecular weight. The values given are an average of several determinations. The iron content was determined on a preparation of specific activity 2.0.

chromatography, the pigment remained with the neutral lipid fraction, and it was found to have a spectrum quite similar to that of coenzyme Q_{10}, which has absorption maxima in the oxidized and sodium borohydride-reduced forms at 275 and 290 nm, respectively. The material, which was not further characterized and is not known to be required for hydroxylation activity, was estimated to be present in the amount of about 0.4 nmol/nmol of hydroxylase polypeptide chain, based on the known extinction coefficient of coenzyme Q_{10} (24). The purified protein contains carbohydrate, which was estimated to be about 5% of the total weight by analysis of acid hydrolysates according to the anthrone method for neutral sugars following column chromatography on Dowex-50 and by determination of glucosamine with the amino acid analyzer.

Amino acid composition. The amino acid content of the ω-hydroxylase is shown in Table II. The most striking feature of the amino acid analysis is the presence of a single cysteine residue per polypeptide chain, in view of the fact that in many nonheme iron proteins four cysteine residues provide sulfur atoms as ligands bound to the iron atom. This enzyme is somewhat richer in hydrophobic amino acid residues than most proteins; the polarity index is 42% by the criteria of Capaldi and Vanderkooi (25), placing it about in the middle of membranous proteins in this respect. In experiments not given, apoenzyme obtained by removal of iron, and apoenzyme from which lipids had been removed by extraction with 1:2 chloroform–methanol were found to have an amino acid content in very close agreement with the values obtained with the holoenzyme. The minimal molecular weight calculated from the amino acid composition is 40,100, which agrees very well with that determined by calibrated polyacrylamide gel electrophoresis.

Sulfhydryl titration. The ω-hydroxylase was treated with DTNB to determine the sulfhydryl content; the results are shown in Table III. Only one sulfhydryl group was detected per polypeptide chain,

TABLE II

AMINO ACID ANALYSIS OF PURIFIED ω-HYDROXYLASE[a]

Amino acid	Number of residues per molecule
CyS(O₃H)	1
Met(O₂)	12
Asx	30
Thr	16
Ser	23
Glx	32
Pro	19
Gly	30
Ala	31
Val	23
Ile	19
Leu	40
Tyr	13
Phe	15
His	14
Lys	18
Trp	6
Arg	18
Total	360

[a] Electrophoretically homogeneous ω-hydroxylase preparations were analyzed as described under Experimental Procedures. The values given are averages of duplicate determinations and are expressed relative to the alanine content.

TABLE III
SULFHYDRYL CONTENT OF ω-HYDROXYLASE[a]

Denaturing agent added	Sulfhydryl content (mol/mol of polypeptide chain)	
	Holoenzyme	Apoenzyme
None	0.96	1.01
Sodium dodecyl sulfate	1.01	0.95
Urea	1.16	1.18
Guanidine hydrochloride	1.01	1.00

[a] A sample of the enzyme (15 nmol) was dialyzed overnight against 0.025 M Tris buffer, pH 7.4, and then incubated with or without denaturing agent added (2% sodium dodecyl sulfate, 6 M urea, or 6 M guanidine hydrochloride, final concentration) for a few minutes at 23°C in reaction mixtures containing 80 μmol of sodium phosphate buffer, pH 8.0, and 1.5 μmol of EDTA. DTNB (0.4 μmol in 0.04 ml of dilute phosphate buffer) was then added to give a final volume of 1.0 ml, and the mixture was incubated at 23°C. The formation of 5-thio-2-nitrobenzoate was followed at 410 nm as a measure of sulfhydryl groups which had been titrated. The reaction was largely over in 15 min and complete by 60 min; the resulting thionitrobenzoate concentration was calculated using an extinction coefficient of 13,600 M^{-1} cm^{-1} (26). Control experiments were included without added enzyme or DTNB. Cysteine standards had the expected sulfhydryl content by this method.

whether the preparation analyzed was the holoenzyme or apoenzyme and whether denaturing agents were present or not. These observations are clearly in accord with the finding of a single cysteic acid residue by amino acid analysis following performic acid oxidation.

Iron removal and reconstitution of the enzyme. Various experiments indicated that removal of iron from the ω-hydroxylase could be accomplished much more readily after chemical reduction. Figure 2 shows the percentage loss of iron and of hydroxylase activity when the enzyme was dialyzed against EDTA or against EDTA in the presence of dithionite. In the absence of the reductant, over 60% of the iron content and activity remained even at 24 h, whereas in the presence of dithionite less than 10% of the activity remained after 4 h. In the latter experiment, iron was also lost extensively but was still present at higher levels than would have been predicted from the loss in activity. The

reason for this discrepancy is not known, but may be due to removal of iron from the active site, followed by nonspecific binding elsewhere in the protein. Such results indicating the more effective removal of ferrous iron from the protein are particularly striking in view of the known greater affinity of EDTA for ferric than ferrous ions (27).

A number of experiments showed that hydroxylase activity could be restored by the addition of ferrous ions to an apoenzyme preparation, whereas ferric ions were ineffective. With ferrous ammonium sulfate, holoenzyme formation was achieved within a few minutes and was dependent on the concentration of the iron salt, as shown in Fig. 3. Maximal activity was obtained with 1.0×10^{-4} M ferrous ions. In other experiments not shown, 2.0×10^{-4} M ferrous ions gave the same effect as 1.0×10^{-4} M, whereas 5.0×10^{-4} M was 60% as effective. Other metals such as Mn^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, and Ca^{2+} gave no restoration of activity when substituted for ferrous ions in such experiments.

The results obtained in some reconstitution experiments in which the activity and iron content were compared are given in Table IV. The apoenzyme obtained in the usual manner contained only a trace of the activity and iron present in the holoenzyme. Reconstitution by the addition of an iron-ascorbate mixture gave a preparation having about 60% of the original activity. Upon dialysis of the reconstituted enzyme the bulk of the activity was lost, whereas holoenzyme dialyzed under the same conditions showed no loss in activity. Obviously, the reconstituted protein is considerably less stable than the enzyme as originally isolated. Furthermore, when the reconstituted and dialyzed enzyme was again put through the reconstitution procedure, only minimal activity was recovered. Other experiments showed similar results when reconstitution was carried out with ferrous ammonium sulfate rather than ferrous ascorbate.

Reversibility of inhibition of ω-hydroxylase by cyanide. As reported previously, the hydroxylase is inhibited by cyanide but not by other anions such as chloride,
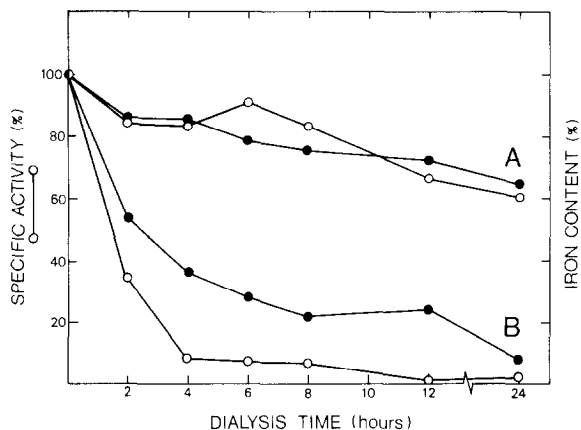
FIG. 2. Hydroxylation activity and iron content of ω-hydroxylase during dialysis. Samples of the ω-hydroxylase (each 1.5 mg of protein in a 2.0-ml volume) were dialyzed at 4°C against 2 liters of 0.2 M Tris buffer, pH 7.4, containing 15 mM disodium-EDTA (Expt A) or a similar solution containing, in addition, 10 mM sodium dithionite (Expt B). The buffers were flushed with nitrogen gas and the flasks were sealed to exclude air during the dialysis. Aliquots were removed and analyzed promptly for hydroxylation activity with octane as substrate, and the iron content was subsequently determined by atomic absorption. The volume of the solution in the dialysis bags did not change significantly during the experiment. The addition of dichloroindophenol to the dialysate at the end of the experiment in which dithionite was included showed that some of the reducing agent still remained.
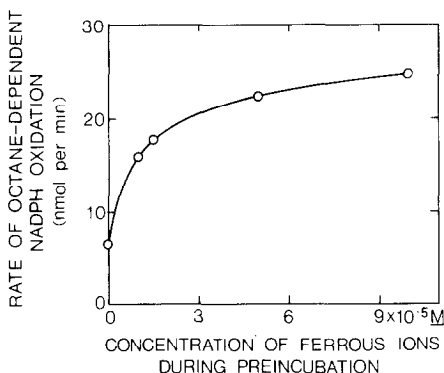

FIG. 3. Reconstitution of hydroxylation activity as a function of ferrous ion concentration. The apohydroxylase (30 μg of protein) was preincubated at 23°C with various concentrations of freshly prepared ferrous ammonium sulfate in the presence of 40 μmol of Tris buffer, pH 7.4, in a final volume of 0.1 ml for 10 min. The sample was then diluted, and the activity was determined promptly by the spectrophotometric assay.

nitrate, sulfate, and thiocyanate (11). The experiments summarized in Table V were carried out to determine whether cyanide reacts irreversibly with some group in the enzyme. Although inhibition was nearly complete at the potassium cyanide concen-

trations used, subsequent removal of the inhibitor restored almost full activity. With the highest cyanide concentration used, over 80% of the initial activity was recovered upon dialysis. Such findings indicate that cyanide reacts reversibly with the enzyme and therefore rule out the type of inhibition seen with some enzymes in which cyanide reacts with a persulfide group with the formation of thiocyanate. For example, Massey and Edmondson (28) have reported such an effect with xanthine oxidase.

Attempts to characterize the iron center by spectral and other methods. As reported earlier, the ω-hydroxylase has a very simple absorption spectrum (12). The enzyme exhibits no peaks in the visible region but has a steadily increasing absorbance from 520 to 360 nm. The only distinguishing characteristic is a very slight shoulder at about 416 nm, which varies in amount with different preparations and may possibly be due to contamination by a trace of hemeprotein. In the ultraviolet region, the maximum is at 283 nm. The spectrum is not significantly altered by the addition of dithionite, hydro-

TABLE IV

RECONSTITUTION OF ω-HYDROXYLASE[a]

Preparation	Specific activity	Iron content (nmol/nmol of poly-peptide)
Holoenzyme	0.9	1.04
Apoenzyme	0.07	0.08
Reconstituted enzyme	0.59	
Reconstituted enzyme, dialyzed	0.15	4.5
Reconstituted enzyme, dialyzed and again reconstituted	0.10	

[a] The reconstitution procedure was carried out as follows. To 400 nmol of Tris buffer, pH 7.4, in 0.8 ml of solution, 0.44 mg (11 nmol) of the apoenzyme (prepared as described under Experimental Procedures) and 0.10 ml of a freshly prepared solution containing 100 nmol of ferric chloride and 200 nmol of ascorbate were added, bringing the final volume to 1.0 ml. This mixture was incubated at room temperature for 4 min, and a 0.1-ml sample was removed and immediately assayed for activity. The remaining solution was dialyzed against 1 liter of 0.05 M Tris buffer, pH 7.4, at 4°C for 18 h. The buffer was flushed with nitrogen during dialysis. The activity and iron content of the reconstituted enzyme were then determined. In a control experiment, a sample of holoenzyme was dialyzed in a similar manner.

gen peroxide, substrates, or cyanide. Furthermore, experiments using the iron-depleted enzyme show that the iron contributes only slightly to the visible spectrum. A variety of attempts to characterize the iron center by epr spectrometry have been carried out in collaboration with Dr. J. Peisach, Albert Einstein University, Dr. G. Palmer, presently at Rice University, and Dr. J. A. Fee and Dr. R. H. Sands, Biophysics Research Division, The University of Michigan. The enzyme exhibits a weak signal at $g = 4.3$ which accounts for only about 16% of the total iron present; the epr spectrum is not significantly changed by the addition of substrates, cyanide, or ferricyanide, but disappears upon reduction of the sample with a few grains of dithionite.

The results described above indicate that neither the absorption spectrum nor the epr spectrum of the ω-hydroxylase has proved useful in studying electron uptake

by the iron atom. Instead, advantage was taken of the spectral properties of rubredoxin, which changes upon oxidation from a colorless to a red form with pronounced peaks at 495 and 377 nm (5). A solution of 300 nmol of rubredoxin in 3.34 ml of 0.05 M Tris buffer, pH 7.3, was placed in an anaerobic cuvette assembly and reduced by the addition of 150 nmol of sodium dithionite via a calibrated syringe. A solution of the ω-hydroxylase (70 nmol of iron; 3.0 mg of protein) in 0.08 ml of buffer was then added from a sidearm under anaerobic conditions, and the extent of reoxidation of rubredoxin was determined at 495 nm. The results indicated that the amount of rubredoxin oxidized was equal to 60% of the ω-hydroxylase iron present. This experiment apparently establishes that the iron atoms of the ω-hydroxylase accept electrons from rubredoxin, but it is not known whether thermodynamic or other considerations prevented complete reduction of the hydroxylase.

Comparison of hydroxylation and epoxidation activities of ω-hydroxylase. The activity of the ω-hydroxylase in hydroxylating a variety of alkanes and fatty acids has been reported earlier (11). More recently, May and Abbott (29, 30) made the interesting finding that the reconstituted enzyme system containing partially purified ω-hydroxylase also catalyzes the epoxidation of

TABLE V

REVERSIBILITY OF CYANIDE INHIBITION[a]

Final cyanide concentration in assay mixture containing undialyzed hydroxylase (M)	Specific activity of ω-hydroxylase	
	Before dialysis	After dialysis
None	0.78	0.80
5×10^{-4}	0.05	0.72
1.0×10^{-3}	0.05	0.72
2.0×10^{-3}	0.01	0.65

[a] Reaction mixtures containing 25 nmol of enzyme, 200 μmol of 0.1 M Tris buffer, pH 7.4, and potassium cyanide at various levels in a final volume of 2.0 ml were incubated for 10 min at 4°C. A 0.1-ml aliquot was removed for assay by the spectrophotometric procedure using octane as substrate, and the remainder was dialyzed for 16 h at 4°C against 6 liters of 0.1 M Tris buffer, pH 7.4. The dialyzed samples, which had undergone no measurable volume change, were then assayed promptly.

alkenes, such as 1,7-octadiene. They observed that the epoxidation reaction resembles alkane hydroxylation in both cyanide sensitivity and pH dependence. The relative activity of the ω-hydroxylase preparations at various stages of purification in the hydroxylation of octane and epoxidation of 1,7-octadiene is shown in Table VI. The disappearance of NADPH was used to measure these reactions, since it has already been established that a 1:1 stoichiometry exists between reduced pyridine nucleotide oxidation and product formation, with either alkane (11) or alkene (30) as the substrate. The ratio of activities toward the two substrates did not change significantly throughout purification of the ω-hydroxylase, taking into account the difficulty of measuring these reactions by the spectral assay in the crude preparations, especially the starting extract. In other experiments not shown, both epoxidation and hydroxylation activities were lost when iron was removed from the enzyme, and the holoenzyme formed upon the addition of iron showed an activity ratio toward octadiene and octane of 1.3. Such findings strongly suggest that the two activities reside in a single protein.

DISCUSSION

The data presented indicate that the ω-hydroxylase is an unusual example of a

mixed function oxidase containing non-heme iron. Since it lacks the acid-labile sulfide typical of ferredoxins and the visible spectra characteristic of ferredoxins and rubredoxins, it clearly differs from these extensively investigated classes of iron–sulfur proteins. A further difference is the presence of a single cysteine residue per polypeptide chain, whereas ferredoxins and rubredoxins have several such residues providing sulfur ligands to the iron atoms. Most of the ligands to the iron in the ω-hydroxylase may be oxygen or nitrogen, which, being more electronegative than sulfur, would be less likely to give rise to the kind of charge-transfer bands seen in the visible spectrum of the rubredoxins (3).

Though activity is lost when iron is removed from the hydroxylase and partially restored when ferrous ions are added to the apoenzyme, the exact role of the iron atom is not .clear. As a working hypothesis, the following sequence of electron transfer reactions in this multienzyme system is proposed:

$$NAD(P)H \xrightarrow{\text{(a)}} \text{reductase} \xrightarrow{\text{(b)}}$$

$$\text{rubredoxin} \xrightarrow{\text{(c)}} \omega\text{-hydroxylase} \xrightarrow{\text{(d)}} O_2.$$

Reactions (a) and (b) have been studied in detail (5, 9), and an interaction between rubredoxin and the reductase has been demonstrated by both spectrophotometric and fluorimetric techniques (10); these two proteins form a complex in a 1:1 ratio. The indirect demonstration in the present paper that reduced rubredoxin is capable of transferring electrons to the ω-hydroxylase under anaerobic conditions supports the involvement of Reaction (c). It has not been possible to obtain direct evidence for the last two steps, however, chiefly due to the lack of spectral evidence for distinct oxidized and reduced forms of the ω-hydroxylase. In contrast, earlier evidence provides indirect support for Step (d) (11). The rates of NADPH oxidation and O_2 consumption were found to be markedly enhanced when the ω-hydroxylase was added to a mixture of the reductase and rubredoxin in the absence of substrate. Presum-
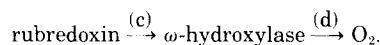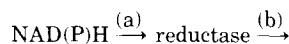
TABLE VI

COMPARISON OF HYDROXYLATION AND EPOXIDATION
ACTIVITIES OF ω-HYDROXYLASE PREPARATIONS

Enzyme preparation	Specific activity		Ratio of activities
	Octadiene epoxidation	Octane hydroxylation	
Streptomycin-treated sonic extract	0.18	0.16	1.1
Ammonium sulfate precipitate (30–35% saturation)	0.54	0.36	1.5
Supernatant fraction after ultracentrifugation	1.02	0.79	1.3
Agarose (A-50m) column eluate	1.58	1.22	1.3
DEAE-cellulose column eluate	1.75	1.23	1.4

ably the autooxidizability of the ω-hydroxylase permits a more rapid flow of electrons from the reduced pyridine nucleotide to oxygen. The addition of substrate to the complete enzyme system gives much higher rates of electron flow, accompanied by oxygen insertion and product formation in Step (d). It may be noted that this reaction sequence involves a unique situation in hydroxylation systems in which one nonheme iron protein, rubredoxin, transfers electrons to another, the ω-hydroxylase. In other well-known hydroxylating enzyme systems with a nonheme iron electron carrier, notably the camphor hydroxylation system of *Pseudomonas putida* (32) and the steroid hydroxylation system of adrenocortical mitochondria (33), the nonheme iron component is a ferredoxin and the monooxygenase is a hemeprotein, cytochrome P-450. On the other hand, electron transfer from reduced pyridine nucleotides to the various forms of cytochrome P-450 in liver microsomes is mediated directly by a flavoprotein without the involvement of a nonheme iron protein (18, 34).

Evidence has been presented that octane hydroxylation and octadiene epoxidation are catalyzed by a single enzyme. They may both be pictured as reactions involving the insertion of one atom of molecular oxygen into substrate and reduction of the other to water, according to the following equation:

Alkane (alkene) + NAD(P)H + H$^+$ + O$_2$
$\rightarrow$ alcohol (epoxide) + NAD(P)$^+$ + H$_2$O.
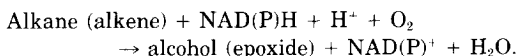
The two reactions have the stoichiometry predicted by this equation (1, 28). It may be noted that with the purified forms of rabbit liver microsomal cytochrome P-450, for example, there is also evidence that a single enzyme catalyzes hydroxylation and epoxidation reactions as well as other transformations (35, 36).

ACKNOWLEDGMENTS

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