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Purification and Properties of the Hydroxylase Component of Methane Monooxygenase

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Methane monooxygenase from *Methylobacterium* sp. strain CRL-26 which catalyzes the oxygenation of hydrocarbons was resolved into two components, a hydroxylase and a flavoprotein. An anaerobic procedure was developed for the purification of the hydroxylase to homogeneity. The molecular weight of the hydroxylase as determined by gel filtration was 220,000, and that determined by sedimentation equilibrium analysis was about 225,000. The purified hydroxylase contained three nonidentical subunits with molecular weights of about 55,000, 40,000, and 20,000, in equal amounts as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, indicating that it is an $\alpha_2\beta_2\gamma_2$ protein. Optical absorption spectra revealed peaks near 408 and 280 nm, and fluorescence spectra revealed emission peaks at 490 and 630 nm. The purified hydroxylase contained 2.8 ± 0.2 mol of iron and 0.5 ± 0.1 mol of zinc per mol of protein but negligible amounts of acid-labile sulfide. The antisera prepared against the hydroxylase showed cross-reactivity with hydroxylase components in soluble extracts from other methanotrophs.

Methanotrophs, organisms capable of utilizing methane as their sole source of carbon and energy, oxidize methane to carbon dioxide via methanol, formaldehyde, and formate. Higgins and Quayle (14) have presented evidence for an oxygenase step in the utilization of methane by showing that the oxygen in the methanol excreted by cell suspensions of *Methanomonas methanooxidans* and *Pseudomonas methanica* is derived exclusively from $^{18}O_2$ and not from water. The role of NADH in the reaction was inferred when oxidation and oxygen consumption catalyzed by the particulate fractions of various methane-utilizing bacteria (10, 21, 22, 27) correlated with NADH oxidation. Tonge et al. (27) partially purified the methane monooxygenase system from particulate fractions derived from the obligate methane-utilizing bacterium *Methylosinus trichosporium* OB3b, and they concluded that three components are required for activity. Also, Colby et al. (5–8) and Dalton (9) have described a multicomponent soluble methane monooxygenase from a strain of *Methylococcus capsulatus* (Bath). Recently we reported that a variety of hydrocarbons are oxidized by cell-free preparations of *Methylobacterium* sp. strain CRL-26 (20). We have resolved methane monooxygenase into two components, a hydroxylase and a flavoprotein. This report describes the purification and properties of the hydroxylase component.

The methane-utilizing organism *Methylobacterium* sp. strain CRL-26 was isolated from soil samples by enrichment culture with methane as a carbon source, as described previously (20). The organism was maintained at 30°C on agar plates containing mineral salts (11) in a dessicator under an atmosphere of methane and air (1:1, vol/vol). Strain CRL-26 was grown on methane at 30°C in 4.0-liter flasks containing 1 liter of mineral salts medium (11) with methane as the sole carbon source (20). Twenty-four-liter cultures were grown on methane at 30°C on mineral salts medium in a 30-liter explosion-resistant fermentor (New Brunswick Scientific Co., Inc., Edison, N.J.). The fermentor was inoculated with 4 liters of a culture grown in shake flasks to an A_{600} of 0.5. A gas mixture of 25% methane (containing 2 to 5% carbon dioxide) and 75% air was continuously sparged through the fermentor at a flow rate of 1 liter/min. Three-hundred-liter cultures were grown on methane at 30°C on mineral salts medium in a 400-liter explosion-resistant fermentor (New Brunswick Scientific Co.). This fermentor was inoculated with a 24-liter methane-grown culture. Cells were harvested with a Sharples centrifuge during the exponential growth phase and were stored at −80°C. Typical yields were 800 g (wet weight) of packed cells. Methane monooxygenase activity was estimated by measuring the epoxidation of propylene as described previously (20). The protein concentration was determined by using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin as a standard.

The purification of the hydroxylase component was carried out at 4°C. Before use, all buffers were repeatedly degassed and flushed with Ar (prepurified by passage over heated BASF catalyst to remove traces of O_2) and were maintained under positive pressure. Frozen cells (100 g) were thawed in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol, 20% glycerol, and DNase (0.05 mg/ml). Cells in suspension at 4°C were broken by a single pass through a French pressure cell (American Instrument Co., Silver Spring, Md.) at 15,000 lb/in^2. Suspensions of broken cells were centrifuged at 40,000 × *g* for 20 min, yielding a soluble fraction designated S(40). The S(40) fraction was centrifuged at 80,000 × *g* for 60 min, yielding particulate and soluble fractions designated P(80) and S(80), respectively. The S(80) fraction contained 98% methane monooxygenase activity. The S(80) fraction was loaded on a DEAE-cellulose column (5 by 50 cm), previously equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol and 20% glycerol (buffer A), at a flow rate of 350 ml/h. The components not bound to DEAE that passed through the column with buffer A were collected and designated fraction 1. The column was then treated with successive batches of 500 ml of buffer A containing 0.15 and 0.3 M NaCl, and the two fractions that eluted with these salt solutions were designated 2 and 3, respectively. Fractions 1, 2, and 3 were then examined for

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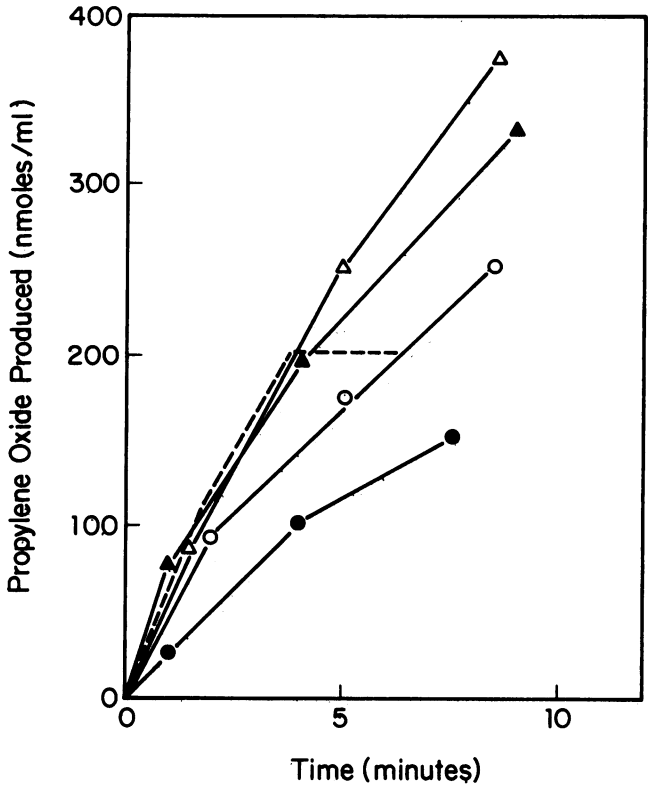

FIG. 1. Effect of NADH concentration on methane monooxygenase activity as measured by the epoxidation of propylene to propylene oxide with the soluble S(80) fraction. The protein concentration was 1.2 mg per assay. NADH concentrations (millimolar) were as follows: 0.312 (– – –), 0.625 (△), 1.25 (▲), 1.8 (○), 3.5 (●).

methane monooxygenase activity; good activity was obtained only when fractions 1 and 3 were combined. For reasons that will be made obvious below, fractions 1 and 3 are designated the hydroxylase and flavoprotein fractions, respectively. Fraction 1, the hydroxylase fraction, was diluted fourfold with 1 liter of 5 mM phosphate buffer (pH 7.0) containing 20% glycerol and 1 mM dithiothreitol and was loaded on a hydroxylapatite column (2.5 by 50 cm), previously equilibrated with 25 mM phosphate buffer (pH 7.0) containing 20% glycerol and 1 mM dithiothreitol, at a flow rate of 150 ml/h. The column was washed with the equilibration buffer, and the hydroxylase was eluted with a linear gradient of 300 ml each of 20 and 200 mM phosphate buffer (pH 7.0) containing 20% glycerol and 1 mM dithiothreitol. The fractions containing hydroxylase activity were pooled, diluted with an equal volume of 10 mM phosphate buffer (pH 7.0) containing 20% glycerol and 1 mM dithiothreitol, and loaded on a quarternary aminoethyl-Sephadex column. After this column was washed, the hydroxylase was eluted with buffer containing 0.15 M NaCl. The fractions containing hydroxylase activity were pooled, concentrated, and stored in liquid nitrogen. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12% gel system was carried out as described by Laemmli (17). Iron was analyzed colorimetrically as described previously (2). Acid-labile sulfide was estimated as described by Chen and Mortenson (4). Metals were also analyzed by inductive coupled plasma atomic emission spectroscopy. Sedimentation experiments were performed with a Spinco model E ultracentrifuge at 58,000 rpm at 4°C according to the procedure of Yphantis (30) with protein concentrations of 3, 6, and 9 mg/ml during the ultracentrifuge analysis. Antisera against the purified hydroxylase component of methane monooxygenase were prepared as described previously (18). The detection of serological cross-reactivity was performed on Ouchterlony double-diffusion plates. The immunoglobulin fractions were separated from immune and normal rabbit sera by successive sodium sulfate fractionation followed by DEAE-cellulose chromatography (16). The partially purified antibody fractions were examined for their ability to inhibit methane monooxygenase as measured by the epoxidation of propylene. Electrophoretic transfer of proteins from SDS-polyacrylamide gel to nitrocellulose and radioautographic detection of these proteins with antibody and radioiodinated protein A were carried out as described by Burnett (3).

Methylobacterium sp. strain CRL-26 grown in a fermentor with methane as the sole carbon source contained mainly soluble methane monooxygenase. Methane monooxygenase activity in the soluble S(80) fraction as measured by the epoxidation of propylene to propylene oxide was inhibited by excess NADH (Fig. 1) or NAD. The concentration of cofactor NADH used during assays was 0.625 mM. Addition of propylene oxide in the reaction mixture before assay inhibited the methane monooxygenase activity. A 50% inhibition of the methane monooxygenase activity was observed at 0.5 mM externally added propylene oxide. The calculated K_i was 0.4 mM for inhibition of methane monooxygenase activity. The enzyme was resolved into two fractions by DEAE-cellulose column chromatography. Component 1 (hydroxylase) and component 3 (flavin adenine dinucleotide: NADH oxidoreductase) were required for methane monooxygenase activity as assayed by the epoxidation of propylene to propylene oxide. The hydroxylase component as separated through a DEAE-cellulose column lost 35% of its activity upon storage at 4°C under aerobic conditions in 72 h. However, anaerobically under similar conditions, the hydroxylase component retained 100% of its activity. The optimal pH for enzyme activity was 7.0. After resolution of methane monooxygenase into two components, a flavoprotein and a hydroxylase, it was discovered that an excess of flavoprotein inhibited the methane monooxygenase activity. During purification of methane monooxygenase, optimal concentrations of each component and the cofactor NADH were supplied. The optimal concentrations of the components were determined by keeping one component at a constant level and varying the concentration of the other component.

The hydroxylase component of methane monooxygenase was purified from the soluble fractions of a cell extract (Table 1). The purified hydroxylase gave a homogeneous single precipitin band on an Ouchterlony double-diffusion plate with antisera prepared against the hydroxylase. By ultracentrifuge analysis, the Schlieren profile of the hydroxylase also revealed a single symmetrical peak. The sedimentation constant ($s_{20,w}$) of the hydroxylase was calculated to be 9.8S, and the molecular weight of the hydroxylase determined by sedimentation equilibrium analysis was about 225,000. This compares with a molecular weight of about 220,000 determined by gel filtration on a Bio-Gel agarose A-1.5 column. By SDS-PAGE, the hydroxylase was shown to have subunits with molecular weights of about 55,000, 40,000, and 20,000 (Fig. 2). Each subunit was present in equal amounts as judged by SDS-PAGE, indicating that the hydroxylase was an $\alpha_2\beta_2\gamma_2$ protein. The UV-visible absorption spectrum of the hydroxylase revealed a peak at around 408 nm (Fig. 3). Upon addition of sodium dithionite, the peak

TABLE 1. Purification of the hydroxylase component of methane monooxygenase[a]

Step	Total vol (ml)	Protein (mg)	Total U	Sp act (nmol/min per mg of protein)	Recovery (%)
Crude extracts	125	2,359	103,796	44	100
DEAE-cellulose column chromatography	287	1,484	96,460	65	97
Hydroxylapatite column chromatography	167	866	91,796	108	88
Quaternary aminoethyl-Sephadex column chromatography	110	400	83,200	208	80

[a] Hydroxylase activity was estimated in the presence of flavoprotein by measuring the epoxidation of propylene to propylene oxide. NADH was the electron donor; O_2 was the oxygen donor.

at 408 nm was not reduced and the optical absorption spectrum remained the same. Reduced spectra did not reveal the absorption peaks at 521 and 551 nm that are characteristic of a heme moiety. The heme content of the purified hydroxylase was negligible. The fluorescence spectra of the purified hydroxylase revealed emission at about 490 and 630 nm (excitation at 408 nm). Determination of acid-labile sulfide in the hydroxylase by colorimetric analysis revealed a negligible amount of sulfide. The purified hydroxylase contained 2.8 ± 0.2 mol of iron per mol of protein as determined by inductive coupled plasma atomic emission spectroscopy and colorimetric analysis. The purified hydroxylase also contained 0.5 ± 0.1 mol of zinc per mol of protein as determined by inductive coupled plasma atomic emission spectroscopy.

Immunoglobulin fractions of antisera prepared against the purified hydroxylase cross-reacted with the hydroxylase and also inhibited methane monooxygenase activity (Fig. 4). Soluble fractions from methane-utilizing *Methylococcus* sp. strain CRL-25, *Methylosinus* sp. strain CRL-16, *Methylobacterium* sp. strain CRL-26, and *Methylobacterium organophilum* XX were subjected to SDS-PAGE. Proteins separated on the polyacrylamide gels were electrophoretically transferred to nitrocellulose filters, and the filters were treated with the immunoglobulin fraction of antisera prepared against hydroxylase. Immunologically similar proteins, exposed by treatment with radioiodinated protein A, were detected in soluble fractions of the organisms listed above at molecular weights of about 60,000 and 40,000 (corresponding to the α and β subunits of the purified hydroxylase) when the nitrocellulose filter was treated with hydroxylase immunoglobulin. The γ subunit did not cross-react with immunoglobulin, probably due to its poor antigenicity.

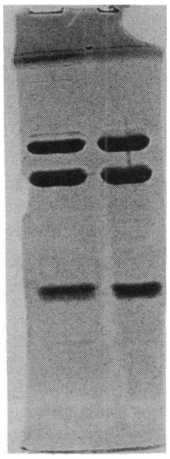

FIG. 2. SDS-PAGE of the purified hydroxylase.

Tonge et al. (26) first reported the separation of a particulate methane monooxygenase from *Methylosinus trichosporium* OB3b into three components. The three-component system catalyzes the hydroxylation of methane to methanol and consists of (i) a soluble carbon-monoxide-binding cytochrome *c* (13,000 molecular weight) containing 1 atom of iron per mol and a variable amount of copper (0.3 to 0.8 atom per mol), (ii) a copper-containing protein (47,000 molecular weight) containing 1 atom of copper per mol, and (iii) a small protein (9,400 molecular weight). The purified enzyme system utilizes ascorbate or methanol (in the presence of methanol dehydrogenase) as an electron donor. NAD(P)H did not serve as an electron donor for the purified system. Tonge et al. (26) have proposed that in *M. trichosporium* OB3b, ascorbate probably directly reduces a carbon-monoxide-binding cytochrome *c* which is essential for monooxygenase activity. The enzyme was not linked directly to NADH, but electrons could be transferred from NADH via an electron transport system to the "physiological" donor. The oxidation of methane by this monooxy-

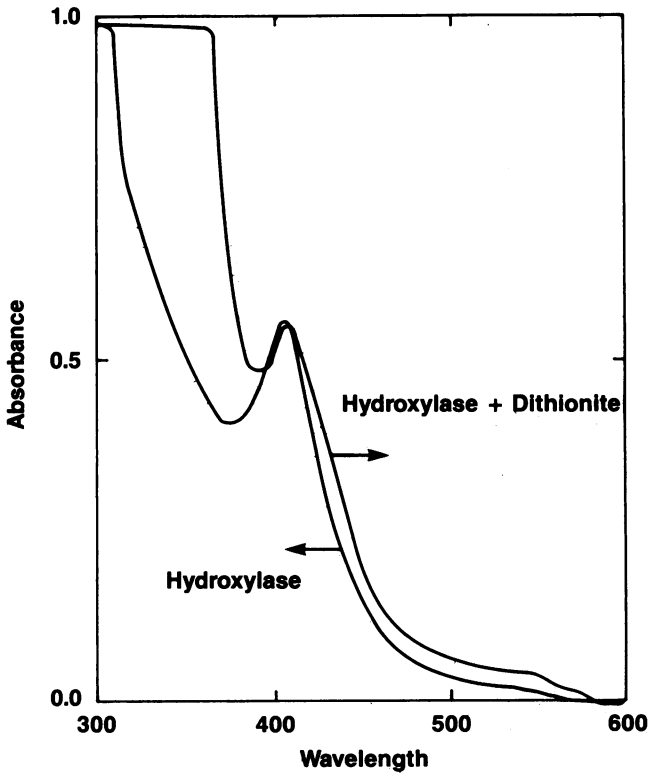

FIG. 3. Absorption spectrum of the purified hydroxylase. The concentration of hydroxylase was 15 mg/ml, and the spectrum was recorded in a Perkin-Elmer spectrophotometer in a 1-ml cuvette.

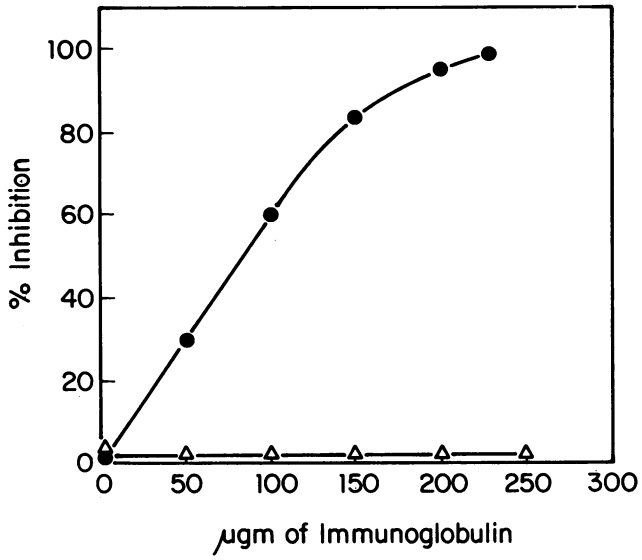

FIG. 4. Inhibition of methane monooxygenase activity by the immunoglobulin fraction from antisera prepared against the purified hydroxylase. Various amounts of immunoglobulin fraction from immune and normal rabbit sera were incubated with a constant amount of purified hydroxylase for about 15 min. The immunoglobulin-treated hydroxylase was used as hydroxylase in the methane monooxygenase assay, and the epoxidation of propylene to propylene oxide was measured. Symbols: ●, hydroxylase treated with the immunoglobulin fraction prepared from immune antisera; △, hydroxylase treated with the immunoglobulin fraction prepared from preimmune antisera.

genase system was inhibited by a variety of metal-binding agents, cyanide, 2-mercaptoethanol, and dithiothreitol.

In contrast to the work of Tonge et al. with *Methylosinus trichosporium* OB3b, Colby and Dalton (6, 7) have reported a soluble methane monooxygenase from *Methylococcus capsulatus* Bath. For this strain, NADH or NADPH supports activity; ascorbate does not serve as an electron donor. This soluble methane monooxygenase was resolved into three required components (A, B and C). Component A has a molecular weight of about 220,000 and subunits with molecular weights of 68,000 and 47,000. It was reported to contain 2 g-atom of iron and acid-labile sulfide per mol. Subsequently, acid-labile sulfide in component A was found to be absent, even though the presence of a 2Fe-2S* center was revealed by core extrusion. Recently, Woodland and Dalton (28, 29) have reported that component A has subunit molecular weights of 55,000, 40,000, and 17,000. Chemical analysis of the purified component A revealed the presence of 2.3 mol of iron and 0.35 mol of zinc per mol of protein. Acid-labile sulfide was not detected in the purified protein. The purified component A has a specific activity of 70 nmol (propylene oxide formed per minute per milligram of protein) and did not reveal an absorption peak in the visible region of the absorption spectrum. Component B is a colorless protein of 15,000 molecular weight, and component C is an iron flavoprotein of 44,000 molecular weight that contains 1 mol of flavin adenine dinucleotide, 2 g-atom of nonheme iron, and 2 mol of acid-labile sulfide per mol of protein. Component B was reported to be regulatory protein, possessing the capacity to convert the enzyme from an oxidase to an oxygenase. Proteins A and C together catalyze the reduction of molecular oxygen to water, a reaction prevented by protein B (12). Component C is the reductase component of the methane monooxygenase and is involved in the transfer of reducing equivalents from NADH to component A.

We have previously reported the epoxidation of alkenes and the hydroxylation of alkanes by a soluble methane monooxygenase present in extracts of *Methylobacterium* sp. strain CRL-26 (20). We have also reported the fractionation of the soluble S(80) extract from strain CRL-26 into three fractions with a DEAE-cellulose column. Fraction 1 was passed through the DEAE-cellulose column; fractions 2 and 3 were eluted from the DEAE-cellulose column with a buffer containing 0.2 and 0.4 M NaCl, respectively. The methane monooxygenase activity was obtained only when fractions 1 and 3 were combined. Fraction 2 stimulated the methane monooxygenase activity as measured by the epoxidation of propylene (20). Subsequently, we have observed that only two fractions are required for activity. This soluble methane monooxygenase has now been resolved into two components, both of which are required for the epoxidation of propylene. One of the components was an iron-containing flavoprotein of 40,000 molecular weight which contained 1 mol of flavin adenine dinucleotide per mol protein and 2 g-atom each of iron and acid-labile sulfide per mol of protein. We have purified the second component, the hydroxylase, of the methane monooxygenase enzyme system. The hydroxylase had a molecular weight of about 230,000 and contained three nonidentical subunits with molecular weights of about 55,500, 40,000, and 20,000. The purified hydroxylase contained about 3.0 mol of iron per mol of protein but a negligible amount of acid-labile sulfide. This suggests the presence of a novel iron-containing prosthetic group in the hydroxylase protein. There is an obligatory requirement for component B, a low (17,000)-molecular-weight regulatory protein in the methane monooxygenase system from *Methylococcus capsulatus* Bath. In contrast, in *Methylobacterium* sp. strain CRL-26, either a regulatory protein is not required for the activity or the function of the regulatory protein is incorporated directly by the hydroxylase protein. The possibility of tight binding of the hydroxylase protein with the regulatory protein and its inability to dissociate during purification cannot be ruled out. However, our SDS-PAGE data do not reveal any contaminating protein with a molecular weight of around 17,000.

In contrast to the results of Tonge et al. (26), recently Stirling et al. (24, 25) have reported that *Methylosinus trichosporium* OB3b contains a soluble methane monooxygenase activity very similar to that of *Methylococcus capsulatus* Bath. The only effective electron donor for this monooxygenase was NAD(P)H. Cross-reactivity between component A from *M. capsulatus* Bath and the similar component from *M. trichosporium* OB3b also was observed. Thus, it appears that all soluble methane monooxygenases are similar. This hypothesis is supported by our demonstration of immunological cross-reactivity of antisera prepared against our purified hydroxylase with the soluble fractions of various methane-utilizing organisms by using radioiodinated protein A to detect the antigen-antibody.

The use of methanotrophic bacteria as biocatalysts for synthesizing industrially important compounds, particularly for oxygenation reactions, has been discussed in recent publications (1, 9, 13). The success of such a process now largely depends on the ability to supply either NADH or a substitute electron donor for the reaction. Future research to determine the mechanism of oxygen insertion into hydrocarbons by methane monooxygenase should lead to an understanding of biocatalytic oxygenation.

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