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Microbial Oxidation of Hydrocarbons: Properties of a Soluble Methane Monooxygenase from a Facultative Methane-Utilizing Organism, *Methylobacterium* sp. Strain CRL-26

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Methylobacterium sp. strain CRL-26 grown in a fermentor contained methane monooxygenase activity in soluble fractions. Soluble methane monooxygenase catalyzed the epoxidation/hydroxylation of a variety of hydrocarbons, including terminal alkenes, internal alkenes, substituted alkenes, branched-chain alkenes, alkanes (C_1 to C_8), substituted alkanes, branched-chain alkanes, carbon monoxide, ethers, and cyclic and aromatic compounds. The optimum pH and temperature for the epoxidation of propylene by soluble methane monooxygenase were found to be 7.0 and 40°C, respectively. Among various compounds tested, only $NADH_2$ or $NADPH_2$ could act as an electron donor. Formate and NAD^+ (in the presence of formate dehydrogenase contained in the soluble fraction) or 2-butanol in the presence of NAD^+ and secondary alcohol dehydrogenase generated the $NADH_2$ required for the methane monooxygenase. Epoxidation of propylene catalyzed by methane monooxygenase was not inhibited by a range of potential inhibitors, including metal-chelating compounds and potassium cyanide. Sulfhydryl agents and acriflavin inhibited monooxygenase activity. Soluble methane monooxygenase was resolved into three components by ion-exchange chromatography. All three compounds are required for the epoxidation and hydroxylation reactions.

Leadbetter and Foster (18, 19) first suggested that the initial oxidation of methane involves an oxygenase reaction. Strong evidence for the involvement of an oxygenase in the oxidation of methane came from the direct isolation of $CH_3{}^{18}OH$ as a product of methane oxidation by cell suspensions of methane-utilizing bacteria (13). Subsequently, there were reports on methane-stimulated $NADH_2$ oxidation and oxygen consumption catalyzed by the particulate fractions derived from the obligate methylotrophs *Methylococcus capsulatus*, Texas strain (28), and *Pseudomonas methanica*, Texas strain (9).

Tonge et al. (37, 38) purified the methane monooxygenase enzyme system from particulate fractions derived from the obligate methane-utilizing bacterium *Methylosinus trichosporium* OB3b and identified three components required for activity. In contrast to this system, Colby et al. (4–7) reported a soluble methane monooxygenase from the obligate methane-utilizing bacterium *Methylococcus capsulatus*, Bath strain, that catalyzed the epoxidation of *n*-alkenes and the hydroxylation of *n*-alkanes.

Stirling et al. (33, 34) reported that *Methylosinus trichosporium* OB3b contained a soluble methane monooxygenase activity similar in many respects to the soluble methane monooxygenase from *Methylococcus capsulatus* Bath. They presented results contrary to the previous report by Tonge et al. (38) in the localization of enzyme activity, electron donor specificity, and inhibition by copper- and iron-chelating compounds.

Recently we discovered the epoxidation of *n*-alkenes and the hydrooxylation of *n*-alkanes by cell suspensions and cell-free particulate fractions of methane-utilizing bacteria (14, 29).

In this report we present properties of a soluble methane monooxygenase from a facultative methane-utilizing bacterium, *Methylobacterium* sp. strain CRL-26.

MATERIALS AND METHODS

Organisms. The facultative methane-utilizing organism *Methylobacterium* sp. strain CRL-26 was isolated from soil samples by enrichment culture, using methane as a carbon source, as described previously (25). The organisms were maintained on mineral salts (10) agar plates in a desiccator under an atmosphere of methane-air (1:1, vol/vol) at 30°C.

Growth of organisms. Organisms were grown in small scale at 30°C in 2.8-liter flasks containing 800 ml

of mineral salts medium (10) with methane (methane-air, 1:1, by volume) as the sole carbon and energy source. Cells were harvested after 24 to 48 h by centrifugation at 10,000 × *g* for 15 min. A larger-scale culture was grown on methane at 30°C in batch culture on mineral salts medium (10) in a 30-liter explosion-resistant fermentor (New Brunswick Scientific Co., Edison, N.J.). The fermentor was inoculated with 2 liters of a culture grown in flasks. A gas mixture of 10% methane, 15% carbon dioxide, and 75% air was continuously sparged into the fermentor. The pH was maintained at around 7.0, and the dissolved oxygen was monitored during fermentation. The amounts of carbon dioxide and methane were analyzed in the off-gas system with an infrared analyzer (Infrared Industries, Inc., Santa Barbara, Calif.) The fermentation room was equipped with an MSA model 511 combustible gas alarm system (Mine Safety Appliances Co., Pittsburgh, Pa.). The automatic safety shutoff was installed on the methane inlet line to the fermentor.

Preparation of cell fraction. Cells (250 g, wet weight) were washed twice with 25 mM potassium phosphate buffer, pH 7.0, and suspended in 250 ml of 25 mM potassium phosphate buffer, pH 7.0, containing 5 mM $MgCl_2$ and DNase (0.05 mg/ml). Cell suspensions at 4°C were disintegrated by a single passage through a French pressure cell (American Instruments Co., Silver Spring, Md.) at 20,000 lb/in^2. Disintegrated cell suspensions were centrifuged at 15,000 × *g* for 15 min to remove unbroken cells. The supernatant solution was then centrifuged at 40,000 × *g* for 60 min, yielding particulate P(40) and soluble S(40) fractions. The soluble S(40) fraction was subsequently centrifuged at 80,000 × *g* for 60 min, yielding particulate P(80) and soluble S(80) fractions. The particulate fractions were suspended in 25 mM potassium phosphate buffer, pH 7.0, containing 5 mM $MgCl_2$ and homogenized at 4°C.

Methane monooxygenase assays. The methane monooxygenase activity was estimated by measuring the epoxidation of propylene to propylene oxide. The reaction mixture contained, in 0.5 ml, 25 μmol of potassium phosphate buffer (pH 7.0), 10 μmol of $NADH_2$, and soluble S(80) fraction. Reaction mixtures were contained in 3.0-ml vials at 4°C. Vials were incubated at 35°C on a reciprocating water bath shaker at 50 oscillations per min. The reaction was started by addition of substrate. The gaseous phase of the vials was removed by vacuum and then replaced with a gas mixture of the substrate and oxygen (1:1, vol/vol).

The rate of oxidation of most of the compounds was measured by estimation of products formed. Specific activities were expressed as nanomoles of product formed per minute per milligram of protein. The oxidation of some compounds was measured by following their utilization from the gas or liquid phase. Samples (2 μl of liquid or 50 μl of gas) were injected into the gas chromatograph at zero time, after 5 and 10 min of incubation of the reaction mixture at 35°C. Specific activities were expressed as nanomoles of substrate utilized per minute per milligram of protein. Appropriate control assays in the absence of $NADH_2$, in the absence of oxygen, and using boiled extracts were carried out with each substrate.

Identification and estimation of products. Various products were identified and estimated by retention time comparisons and co-chromatography with authentic standards, using flame-ionization gas chromatography. A stainless-steel column (12 ft by ⅛ in. [ca. 366 by 0.32 cm]) packed with 10% Carbowax 20 M on an 80/100 Chromosorb W or Porapak Q column or a stainless-steel column (6 ft by ⅛ in. [ca. 183 by 0.32 cm]) packed with Carbopack C–0.1% SP-1000 or with GP 5% SP-1200–1.75% Bentone 34 on 100/120 Supelcoport was used. The column temperature was maintained isothermally between 80 and 200°C, with helium carrier gas flow rates of 20 to 40 ml/min. The amount of product formed was estimated from peak areas, using standard graphs which were constructed with authentic compounds.

Duplicate measurements were carried out for each substrate. Protein concentrations in cellular fractions were estimated with Folin-Ciocalteu reagent (20), using bovine serum albumin as a standard. Formaldehyde was estimated colorimetrically by the Hantzseh reaction (23).

Stoichiometry of propylene oxidation. The reaction mixture (3.0 ml) contained 2.8 ml of 25 mM potassium phosphate buffer, pH 7.0, 2 μmol of NADH, and 10 μmol of propylene. The reaction was started by injecting 200 μl of soluble S(80) fraction. The amount of oxygen consumed during the reaction time was determined polarographically. The propylene oxide formed was estimated by gas chromatography. A correction was made for endogenous oxygen consumption. NADH oxidized was measured spectrophotometrically. A correction was made for endogenous NADH oxidation.

Resolution of methane monooxygenase components. Soluble S(80) fraction (20 ml) was loaded on a DEAE-cellulose column (0.9 by 30 cm) equilibrated with 25 mM potassium phosphate buffer (pH 7.0) containing 5 mM $MgCl_2$ and 5 mM dithiothreitol (buffer A). Proteins not adsorbed to DEAE-cellulose (fraction A) were eluted with buffer A. The column was then eluted with successive 25-ml batches of buffer A containing 0.2 M NaCl and 0.5 M NaCl. Fractions from the 0.2 M NaCl eluate (fraction B) having a dark brown color were pooled, and those from the 0.5 M NaCl eluate (fraction C) with a yellow color were also pooled.

Chemicals. Gaseous hydrocarbons (methane, ethane, propane, butane, ethylene, propylene, 1-butene, butadiene, isobutylene, *cis*-but-2-ene, *trans*-but-2-ene, chloromethane, bromomethane, fluoromethane, dimethyl ether, isobutane, and ethylene oxide) were obtained from Matheson Gas Products (East Rutherford, N.J.). Propylene oxide, 1,2-epoxybutane, 1,2-epoxybutene, *cis*-2-buten-1-ol, primary alcohols (C_1 to C_8), secondary alcohols (C_3 to C_8), cyclohexane, toluene, cyclohexanol, benzyl alcohol, isobutanol, *tert*-butanol, butylether, *trans*-2,3-epoxybutane, *cis*-2,3-epoxybutane, and *trans*-2-butene-1-ol were from Pfaltz and Bauer Research Chemical Co. (Stanford, Conn.). Thiourea, thiosemicarbazide, iodoacetamide, 8-hydroxyquinoline, acriflavin, *N*-ethylmaleimide, *p*-hydroxymercuribenzoate, α,α-bipydridyl, $NADH_2$, $NADPH_2$, and dithiothreitol were purchased from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Previously we reported that methylotrophic bacteria, including *Methylobacterium* sp. strain CRL-26, grown in shake flasks with methane (methane-air, 1:1, by volume) as sole carbon

TABLE 1. Hydroxylation of alkanes by soluble methane monooxygenase from *Methylobacterium* sp. strain CRL-26[a]

Substrate	Product	Sp act (nmol/min per mg of protein)
Methane	Methanol	93
Ethane	Ethanol	64
Propane	1-Propanol	15
	2-Propanol	22
Butane	1-Butanol	43
	2-Butanol	25
Pentane	1-Pentanol	21
	2-Pentanol	45
Hexane	1-Hexanol	39
	2-Hexanol	21
Heptane	1-Heptanol	12
	2-Heptanol	50
Octane	1-Octanol	2.5
	2-Octanol	17

[a] Reactions were carried out as described in the text. Products were identified and estimated by gas chromatography retention time comparisons and co-chromatography with authentic standards.

source mainly contained particulate methane monooxygenase activity (29). Now we have discovered that *Methylobacterium* sp. strain CRL-26 obtained by growth of organisms in a 30-liter fermentor contained methane monooxygenase activity mainly in the soluble S(80) fraction. Soluble S(80) fraction was used as the source of methane monooxygenase for examining the substrate specificity and for analysis of products.

Oxidation of *n*-alkanes by soluble methane monooxygenase. Methane monooxygenase catalyzed $NADH_2$- and oxygen-dependent hydrooxylation of *n*-alkanes (C_1 to C_8 tested). Methane was oxidized at the highest rate. C_2 to C_7 alkanes were oxidized at comparable rates, but the rate of oxidation of octane was considerably lower. Both primary and secondary alcohols were produced from the oxidation of C_3 to C_8 alkanes (Table 1).

Oxidation of substituted alkane derivatives. The oxidation of substituted alkane derivatives was measured by following the utilization of substrate. Among monohalogenated methane derivatives, bromomethane and chloromethane were oxidized more rapidly than fluoromethane. Formaldehyde was detected as the product of chloromethane, bromomethane, and fluoromethane oxidation. 1-Nitropropane and 1-bromobutane were oxidized more rapidly than were 2-nitropropane and 2-bromobutane. The branched-chain alkane isobutane was oxidized to isobutanol and tertiary butanol. Trichloromethane oxidized more slowly than did chloromethane and dichloromethane. Carbon monoxide was oxidized to carbon dioxide by soluble methane monooxygenase (Table 2).

Oxidation of alkenes. Methane monooxygenase catalyzed the epoxidation of the terminal alkenes ethylene, propylene, 1-butene, and butadiene to the corresponding 1,2-epoxides. Propylene was oxidized at the highest rate. Internal alkenes such as *trans*-but-2-ene and *cis*-but-2-ene were also oxidized. A mixture of *trans*-2,3-epoxybutane and *trans*-2-buten-1-ol was produced from *trans*-but-2-ene, indicating that both the internal double bond and the terminal methyl group were oxidized. Similarly, *cis*-but-2-ene was oxidized to *cis*-2,3-epoxybutane and *cis*-2-buten-1-ol. The branched-chain alkenes isobutylene and isoprene were oxidized to 1,2-epoxyisobutene and 1,2-epoxyisoprene, respectively (Table 3). Substituted alkenes such as 2-methyl-1-butene, 2-methyl-2-butene, 1-bromo-1-butene, and 2-bromo-2-butene were also oxidized. The rate of oxidation was estimated by measuring the rate of utilization of substrate (Table 3).

Oxidation of ethers. Dimethyl ether oxidation by soluble methane monooxygenase was followed by measuring product formation. Methanol and formaldehyde were detected as products of dimethyl ether oxidation. Butyl ether was also oxidized by soluble methane oxygenase. The rate of butyl ether oxidation was measured by following the rate of butyl ether utilization from the reaction mixture (Table 4).

Oxidation of cyclic and aromatic compounds. Cyclohexane was oxidized to cyclohexanol, and toluene was oxidized to benzyl alcohol and

TABLE 2. Oxidation of substituted alkane derivatives by soluble methane monooxygenase from *Methylobacterium* sp. strain CRL-26[a]

Substrate	Product	Sp act (nmol/min per mg of protein)
Chloromethane	Formaldehyde	44
Bromomethane	Formaldehyde	48
Fluoromethane	Formaldehyde	19
Nitromethane	ND[b]	12
Nitroethane	ND	18
1-Nitropropane	ND	50
2-Nitropropane	ND	19
1-Bromobutane	ND	49
2-Bromobutane	ND	10
Isobutane	Isobutanol	34
	Tertiary butanol	40
Dichloromethane	ND	40
Trichloromethane	ND	21
Carbon monoxide	Carbon dioxide	30

[a] Reactions were carried out as described in the text. Products were identified and estimated by gas chromatography retention time comparisons and co-chromatography with authentic standards.

[b] ND, Product not identified.

TABLE 3. Epoxidation of alkenes by soluble methane monooxygenase from *Methylobacterium* sp. strain CRL-26[a]

Substrate	Product	Sp act (nmol/min per mg of protein)
Ethylene	Ethylene oxide	55
Propylene	Propylene oxide	100
But-1-ene	1,2-Epoxybutane	87
Butadiene	1,2-Epoxybutene	75
Isobutylene	1,2-Epoxyisobutene	95
cis-But-2-ene	*cis*-2,3-Epoxybutane	22
	cis-2-Buten-1-ol	15
trans-But-2-ene	*trans*-2,3-Epoxybutane	25
	trans-2-Buten-1-ol	18
2-Methyl-1-butene	ND[b]	42
2-Methyl-2-butene	ND	16
1-Bromo-1-butene	ND	83
2-Bromo-2-butene	ND	30
Isoprene	1,2-Epoxyisoprene	38

[a] Reactions were carried out as described in the text. Products were identified and estimated by gas chromatography retention time comparisons and co-chromatography with authentic standards.

[b] ND, Product not identified.

cresol. Benzene was hydroxylated to phenol (Table 4).

Electron donor specificity. A number of electron donors were unable to replace $NAD(P)H_2$ as cofactor. Various cofactor systems were examined for epoxidation of propylene. When $NADPH_2$ replaced $NADH_2$, about 70% of the monooxygenase activity was observed. It has been reported by Tonge et al. (37, 38) that ascorbate in the presence of CO-binding cytochrome *c* could replace $NADH_2$ as an electron donor for the particulate methane monooxygenase of *Methylosinus trichosporium* OB3b. Ascorbate in the absence or presence of CO-binding cytochrome *c* could not replace $NADH_2$ as an electron donor for our enzyme (Table 5).

It has been reported that methanol in the presence of methanol dehydrogenase could act as an electron donor for methane monooxygenase systems (37, 38). Methanol in the presence or absence of methanol dehydrogenase could not replace $NADH_2$ as an electron donor for our enzyme (Table 5).

Formaldehyde in the presence or absence of $NAD(P)^+$ could not act as an electron donor for the soluble methane monooxygenase of *Methylobacterium* sp. strain CRL-26, indicating the absence of $NAD(P)^+$-linked formaldehyde dehydrogenase activity to regenerate the reduced cofactor required for the monooxygenase. Formate in the presence of NAD^+ acts as an excellent electron donor for the soluble methane monooxygenase of *Methylobacterium* sp. strain CRL-26 due to the presence of NAD^+-linked formate dehydrogenase in the soluble extracts (Table 5). Secondary butanol in the presence of secondary alcohol dehydrogenase and NAD^+ also acted as an electron donor.

Effect of pH and temperature on soluble methane monooxygenase. Activity for the epoxidation of propylene by soluble methane monooxygenase was observed in the pH range between 6 and 9, the optimum pH was about 7 (Fig. 1). The optimum temperature was about 40°C (Fig. 2).

Effect of inhibitors on soluble methane monooxygenase. It has been reported that the oxidation of methane by cell suspensions of methane-utilizing bacteria was inhibited by various metal-binding or metal-chelating agents (14, 24, 31, 38). The hydroxylation of *n*-alkanes and the epoxidation of *n*-alkenes catalyzed by particulate methane monooxygenase were also inhibited by various metal-binding compounds such as α,α-bipyridyl, 1,10-phenanthroline, potassium cyanide, thiosemicarbazide, thiourea, and 8-hydroxyquinoline (29).

A range of inhibitors were tested on the epoxidation of propylene by soluble methane monooxygenase (Table 6). Among metal-binding or metal-chelating agents, 1,10-phenanthroline and α,α-bipyridyl showed some inhibition. Thiosemicarbazide, thiourea, potassium cyanide, imidazole, and 8-hydroxyquinoline were not inhibitory. Among sulfhydryl agents, iodoacetamide and 5,5′-dithiobis-2-nitrobenzoate inhibited the epoxidation of propylene.

Stoichiometry. The stoichiometry of propylene oxide formed, $NADH_2$ oxidized, and oxygen consumed during oxidation of propylene by soluble methane monooxygenase was approxi-

TABLE 4. Oxidation of ethers and cyclic and aromatic compounds by soluble methane monooxygenase from *Methylobacterium* sp. strain CRL-26

Substrate	Product[a]	Sp act (nmol/min per mg of protein)[b]
Dimethyl ether	Methanol	25
	Formaldehyde	10
Butyl ether	ND	34
Cyclohexane	Cyclohexanol	36
Toluene	Benzyl alcohol	22
	Cresol	15
Benzene	Phenol	20

[a] Products of reactions were identified and estimated by gas chromatography retention time comparisons and co-chromatography with authentic standards. ND, Product not identified.

[b] Specific activities are expressed as the rate of product formation in S(80) fractions.

TABLE 5. Effect of various electron donor systems on soluble methane monooxygenase of *Methylobacterium* sp. strain CRL-26

Electron donor	Concn (mM)	Sp act (nmol/min per mg of protein)[a]
$NADH_2$	2.5	100
$NADPH_2$	2.5	70
Sodium L-ascorbate	5.50	0
Sodium L-ascorbate + co-binding cytochrome[b]	5,200 μg	0
Methanol	5.50	0
Methanol + methanol dehydrogenase[b]	5 + 100 μg	0
Formaldehyde	2.5	0
Formaldehyde + NAD^+	2.5 + 2.5	0
Formaldehyde + $NADP^+$	2.5 + 2.5	0
Formate	5	0
Formate + NAD^+	5 + 2.5	240
2-Butanol + NAD^+ + yeast secondary alcohol dehydrogenase[b]	5 + 2.5 + 100 μg	210

[a] Specific activities are expressed as the rate of propylene oxide formed.
[b] Methanol dehydrogenase from *M. methanica* was purified as described previously (26). Secondary alcohol dehydrogenase from *Pichia* sp. was purified as described earlier (28). Carbon monoxide-binding cytochrome *c* from *Methylobacterium* sp. was purified as described previously by Tonge et al. (38).

mately 1:1:1, indicating a monooxygenase-catalyzed reaction.

Substrate competition. The presence of a hydroxylation reaction substrate (i.e., methane) inhibited (about 65%) the epoxidation of propylene by soluble methane monooxygenase.

Resolution of soluble methane monooxygenase. Soluble methane monooxygenase was resolved into three fractions by DEAE-cellulose chromatography. Fraction A does not bind to the column. Fractions B and C were eluted from the DEAE-cellulose column with 0.2 and 0.5 M NaCl, respectively. All three fractions were required for maximum activity (Table 7). About 50% activity was obtained with fractions A and C, compared with 100% activity with fractions A, B, and C. Fractions A and B are dark brown and fraction C is yellow after separation by ion-exchange chromatography.

DISCUSSION

Van der Linden demonstrated the production of 1,2-epoxides from 1-octene by cell suspensions of *Pseudomonas* sp. that had been grown on heptane (39). Epoxides were not detected as products of alkane metabolism and were not oxidized by *Pseudomonas* sp. Van der Linden (39) postulated that the enzyme system that forms epoxides may be the same as the system that catalyzes the initial oxidation of alkanes. Cardini and Jurtshuk (3) isolated a cell-free extract from a *Corynebacterium* sp. strain which carried out the epoxidation of 1-octene to epoxyoctane in addition to the hydroxylation of octane to octanol (3).

McKenna and Coon (22) demonstrated the hydroxylation of alkanes (C_6 to C_{12}) by an enzyme system derived from *Pseudomonas oleovorans*. Subsequently, it was reported that the enzyme system from *P. oleovorans* catalyzed the epoxidation of 1-alkenes (C_6 to C_{12}) in addition to the hydroxylation reactions (1, 21). The enzyme systems from *P. oleovorans* (22) and *Corynebacterium* sp. (3) catalyzed the epoxidation of C_6 to C_{12} alkenes.

Hydroxylation of methane by cell-free particulate fractions was demonstrated in obligate methylotrophs (9, 16, 29, 30). $NADH_2$ and oxygen were required for the hydroxylation reaction. Hydroxylation of methane by particulate fractions was inhibited by metal-chelating or metal-binding compounds (15, 16, 27, 29, 30).

The particulate methane monooxygenase from *Methylosinus trichosporium* OB3b was fractionated into two proteins: a copper-containing protein with molecular weight 47,000 and a small-molecular-weight protein (9,400 molecular weight). These two proteins together with a soluble CO-binding cytochrome *c* (molecular weight, 13,000) are required for the hydroxylation of methane. The purified enzyme system required ascorbate or, in the presence of methanol dehydrogenase, methanol as an electron donor. $NAD(P)H_2$ could not act as an electron donor. It was suggested that the immediate electron donor is the CO-binding cytochrome *c*, which in the purified enzyme system can be reduced by ascorbate but not by $NAD(P)H_2$. The purified methane monooxygenase was highly sensitive to low concentrations of a variety of metal-chelating agents, cyanide, 2-mercaptoethanol, and dithiothreitol (38).

In contrast to the particulate methane monooxygenase, Colby et al. (4–7) reported a soluble

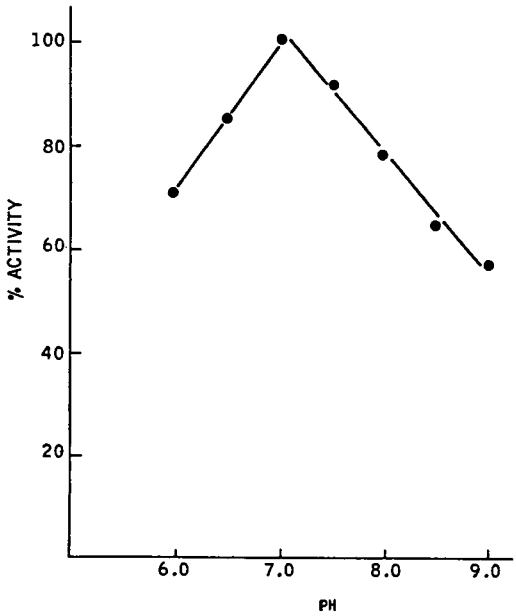

FIG. 1. Effect of pH on epoxidation of propylene by soluble methane monooxygenase from *Methylobacterium* sp. strain CRL-26.

methane monooxygenase system from *Methylococcus capsulatus* Bath, which catalyzed the $NADPH_2$- and oxygen-dependent oxygenation of alkanes, alkenes, ethers, and alicyclic, aromatic, and heterocyclic compounds. Ascorbate or methanol in the presence of methanol dehydrogenase could not act as an electron donor (4, 5, 7). The soluble methane monooxygenase from *Methylococcus capsulatus* Bath was resolved into three components by ion-exchange chromatography. Protein A, with a molecular weight of 220,000, contained 2 g-atoms of iron and acid-labile sulfide per mol of protein. Protein C had a molecular weight of 44,000. It contained 1 mol of flavin adenine dinucleotide and 2 g-atoms each of nonheme iron and acid-labile sulfur. Protein B is a colorless protein with a molecular weight of 15,000. All three proteins were required for activity (5). Soluble methane monooxygenase from *Methylococcus capsulatus* Bath was not inhibited by various metal-chelating agents; however, it was strongly inhibited by ethylene (in air) and 8-hydroxyquinoline.

Stirling et al. (33, 34) reported the presence of soluble methane monooxygenase activity from *Methylosinus trichosporium* OB3b and presented results contrary to the earlier report by Tonge et al. (38) with regard to the localization of activity in cell-free extracts, nature of electron donor, and inhibition of enzyme by inhibitors. The substrate specificity of soluble methane monooxygenase from *Methylosinus trichosporium* OB3b was found to be similar to that from *Methylococcus capsulatus* Bath.

We have reported the epoxidation of alkenes (14, 15) and the hydroxylation of alkanes (30) to secondary alcohols by cell suspensions of methane-utilizing bacteria. Secondary alcohols were further oxidized to methylketones by cell suspensions. The epoxidation of alkenes and the hydroxylation of alkanes were catalyzed cell-free particulate fractions derived from these methylotrophic bacteria (29). We also presented evidence indicating that the epoxidation and hydroxylation reactions were catalyzed by a similar metal-containing monooxygenase. The epoxidation of alkenes and the hydroxylation of alkanes catalyzed by the particulate fractions were inhibited by various metal-chelating agents.

Now we have discovered that, depending upon conditions of growth, the methane monooxygenase activity can be obtained in the soluble fraction from a facultative methane-utilizing organism, *Methylobacterium* sp. strain CRL-26. Thus, cells grown in a fermentor with continuous gassing with methane contained soluble methane monooxygenase. In contrast, cells grown in shake flask cultures with methane (methane-air, 50:50, vol/vol) contained mainly particulate methane monooxygenase activity as described previously (29). It has been demonstrated that *Methylosinus trichosporium* OB3b (11) and *Methylomonas* sp. (8) grown in continu-

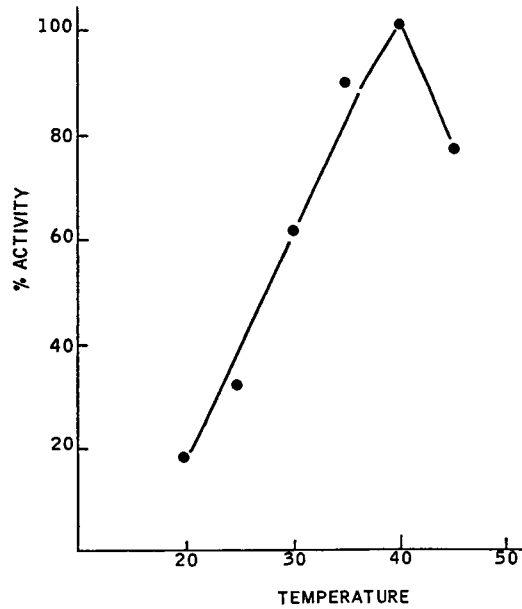

FIG. 2. Effect of temperature on epoxidation of propylene by soluble methane monooxygenase from *Methylobacterium* sp. strain CRL-26.

TABLE 6. Effect of metal binding and sulfhydryl inhibitors on soluble methane monooxygenase

Inhibitor	Concn (mM)	Activity (%)	Inhibition[a] (%)
Control	1	100	0
Thiosemicarbazide	1	108	0
1,10-Phenanthroline	1	67	33
Potassium cyanide	1	108	0
α,α-Bipyridyl	1	74	24
Thiourea	1	100	0
Imidazole	1	130	0
8-Hydroxyquinoline	1	117	0
N-Ethylmaleimide	1	102	0
Iodoacetamide	1	20	80
5,5'-Dithiobis-2-nitrobenzoate	1	38	62
Acriflavin	0.1	71	29
p-Hydroxymercuribenzoate	0.1	100	0

[a] The uninhibited rate of propylene oxidation was 100 nmol of propylene oxide produced/min per mg of protein. Reactions were carried out as described in the text. All inhibitors were incubated in the reaction mixture at 0°C for 15 min. Reactions were started by gassing the vials with propylene.

TABLE 7. Resolution of soluble methane monooxygenase into three fractions by DEAE-cellulose chromatography

Fraction	Sp act (nmol/min per mg of protein)[a]
Before DEAE	95
A	0
B	0
C	0
A + B (1:1 by mg of protein)	0
A + C (1:1 by mg of protein)	65
B + C (1:1 by mg of protein)	0
A + B + C (1:1:1 by mg of protein)	125

[a] Activity was measured by estimating propylene oxide production from propylene, using $NADH_2$ as an electron donor as described in the text.

ous culture in a fermentor contained very few intracytoplasmic membranes compared with shake flask-grown cells. Furthermore, considerable amounts of storage material were present when cells were grown in the fermentor compared with shake flask-grown cells.

Recently, Scott et al. (32) demonstrated that, in *Methylosinus trichosporium* OB3b during oxygen-limited growth conditions in shake flasks, culture possess extensive intracytoplasmic membranes and contain particulate methane monooxygenase activity. However, organisms grown under high oxygen tension possess very few intracytoplasmic membranes and contain mainly soluble methane monooxygenase activity.

Soluble methane monooxygenase from *Methylobacterium* sp. strain CRL-26 catalyzed the epoxidation-hydroxylation of alkenes, alkanes, carbon monoxide, ethers, and cyclic, alicyclic, and aromatic compounds.

Particulate methane monooxygenase activity from *Methylobacterium* sp. strain CRL-26 was inhibited by metal-chelating or metal-binding compounds (29). Soluble methane monooxygenase activity was not inhibited by various metal-binding or metal-chelating compounds or by potassium cyanide, indicating a pattern of inhibition similar to that of methane monooxygenase from *Methylococcus capsulatus* Bath. However, 8-hydroxyquinoline, a potent inhibitor of the soluble methane monooxygenase from *Methylococcus capsulatus* Bath, did not inhibit the soluble methane monooxygenase from *Methylobacterium* sp. strain CRL-26. Sulfhydryl inhibitors (e.g., iodoacetamide, 5,5'-dithiobis-2-nitrobenzoate) and acriflavin inhibited the monooxygenase activity from *Methylobacterium* sp. Absence of inhibition of soluble methane monooxygenase activity by metal-chelating compounds suggests that inhibition of particulate methane monooxygenase may be due to the inhibition of membrane-bound electron carrier protein by metal-chelating compounds.

Recently, Higgins et al. (12) demonstrated the biotransformation of a variety of hydrocarbons by cell suspensions of *Methylosinus trichosporium* OB3b grown on methane, indicating the broad range of compounds oxidized by methane-utilizing organisms, presumably due to the action of methane monooxygenase.

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