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Oxidation of C_1 Compounds by Particulate Fractions from *Methylococcus capsulatus*: Distribution and Properties of Methane-Dependent Reduced Nicotinamide Adenine Dinucleotide Oxidase (Methane Hydroxylase)

DOUGLAS W. RIBBONS

Department of Biochemistry, University of Miami School of Medicine and Howard Hughes Medical Institute, Miami, Florida 33152

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Cell-free particulate fractions of extracts from the obligate methylotroph *Methylococcus capsulatus* catalyze the reduced nicotinamide adenine dinucleotide (NADH) and O_2-dependent oxidation of methane (methane hydroxylase). The only oxidation product detected was formate. These preparations also catalyze the oxidation of methanol and formaldehyde to formate in the presence or absence of phenazine methosulphate with oxygen as the terminal electron acceptor. Methane hydroxylase activity cannot be reproducibly obtained from disintegrated cell suspensions even though the whole cells actively respired when methane was presented as a substrate. Varying the disintegration method or extraction medium had no significant effect on the activities obtained. When active particles were obtained, hydroxylase activity was stable at 0 C for days. Methane hydroxylase assays were made by measuring the methane-dependent oxidation of NADH by O_2. In separate experiments, methane consumption and the accumulation of formate were also demonstrated. Formate is not oxidized by these particulate fractions. The effects of particle concentration, temperature, pH, and phosphate concentration on enzymic activity are described. Ethane is utilized in the presence of NADH and O_2. The stoichiometric relationships of the reaction(s) with methane as substrate were not established since (i) the presumed initial product, methanol, is also oxidized to formate, and (ii) the contribution that NADH oxidase activity makes to the observed consumption of reactants could not be assessed in the presence of methane. Studies with known inhibitors of electron transport systems indicate that the path of electron flow from NADH to oxygen is different for the NADH oxidase, methane hydroxylase, and methanol oxidase activities.

Methylococcus capsulatus was first isolated by Foster and Davis (4) and described as a thermotolerant methylotroph. Like many other methane oxidizing bacteria, this species is only able to grow in the presence of methane (or methanol) and is thus apparently an obligate methylotroph (4, 26). The metabolic sequences that enable *M. capsulatus* to assimilate carbon from methane (or methanol) as sole carbon source have been elucidated from the experiments of Quayle and Kemp and their collaborators (8–10, 15). Formaldehyde formed from the oxidation of methane is assimilated by condensation with ribulose 5-phosphate to give a hexul-3-ose 6-phosphate (8) which is isomerized to fructose 6-phosphate. The acceptor molecule, ribulose 5-phosphate, is regenerated by a transaldolase-transketolase series of reactions, so that net synthesis of cellular constituents is achieved. The isotopic data from Calvin-type experiments and detailed estimations of the distribution of radioactivity from $[^{14}C]CH_3OH$ into glucose 6-phosphate isolated from the hot alcohol-soluble fraction of the cells are entirely in accord with this proposition, as are the enzymic activities found in extracts of cells. However, the reactions involved in the initial transformation of methane to formaldehyde are not well defined (7, 13, 15, 16, 18). As anticipated (11), methane is oxidized by methylotrophs to methanol by an oxygenative reaction (7). The incorporation of oxygen from $^{18}O_2$, but not from $H_2{}^{18}O$, into the methanol formed when washed cell suspensions of two other obligate methylotrophs (*Pseudomonas methanica* and *Methanomonas methanooxidans*) were oxidiz-

ing methane provided unequivocal evidence for the proposal made earlier by Leadbetter and Foster (11) that an oxygenative attack of methane was the first step in its oxidation and ultimate assimilation.

All methane-oxidizing bacteria so far described possess extensive intracytoplasmic membranes (2, 14, 16, 19, 20) which are arranged in one of two general ways. The arrangements found are of taxonomic value (26). Type I membrane systems extend throughout the cytoplasm as stacks and are characteristic of the proposed genera *Methylomonas* (2, 15, 26), *Methylobacter* (2, 26), and *Methylococcus* (2, 14, 16, 20). Type II membrane systems appear at the periphery of the cell and are usually concentric with the cytoplasmic membrane; they are found in the genera *Methylocystis* and *Methylosinus* (2, 19, 26). Since these membrane systems can account for 40 to 60% of the cellular volume, we prepared cell-free preparations of them to study their possible role in the oxidation of C_1 compounds (18). A particulate fraction was isolated from disintegrated suspensions of *M. capsulatus* which catalyzed a methane-dependent oxidation of reduced nicotinamide adenine dinucleotide (NADH) by oxygen (methane hydroxylase). This enzymic activity was difficult to obtain reproducibly from cells which respire well when methane is provided as substrate. Ferenci (3) has recently obtained similar particulate fractions from *P. methanica* that catalyze a methane-dependent oxidation of NADH. This report summarizes some of the properties of the methane hydroxylase system and shows that methanol and formaldehyde are also oxidized with oxygen reduction without the requirements of auxilliary electron donors or acceptors. The methanol and formaldehyde oxidase activities are distinct from the soluble dehydrogenase activities previously obtained from this species by Patel et al. (12, 13) and ourselves (24), in that phenazine methosulfate is not an obligatory component of the assay.

MATERIALS AND METHODS

Chemicals. Methane (99.8%), ethane (99.5%), and propane (99%) were purchased from Phillips Petroleum Co., Bartlesville, Okla. Methane (98%) used for cultivation purposes was obtained from Matheson Gas Products, Morrow, Ga. Coenzymes and the inhibitors, urethane, hydroxyquinoline *N*-oxide, antimycin A, *o*-phenanthroline, and acriflavin were provided by Sigma Chemical Co., St. Louis, Mo. All other reagents used were of the highest purity available.

Cultivation methods. The strain of *M. capsulatus* (Texas) used was that originally described by Foster and Davis (4) and kindly provided by J. R. Quayle and W. H. Kingham. The coccus was maintained as slopes in McCartney screw-cap bottles fitted with serum caps lined with silicone rubber gaskets which were exposed to the atmosphere provided. This allows free diffusion of the gases provided (approximately 20% methane in air) into the culture bottles and maintains a convenient sterile seal in the humid conditions that ensue during incubation in standard anaerobic jars. The composition of the medium was (g/liter): $Na_2HPO_4 \cdot 7H_2O$, 1.6; KH_2PO_4, 0.4; NH_4Cl, 0.4; $MgSO_4 \cdot 7H_2O$, 0.15; and (mg/liter): $FeCl_3 \cdot 6H_2O$, 16.7; $CaCl_2 \cdot 2H_2O$, 0.66; $ZnSO_4 \cdot 7H_2O$, 0.18; $CuSO_4 \cdot 5H_2O$, 0.16; $MnSO_4 \cdot H_2O$, 0.11; $CoCl_3 \cdot 6H_2O$, 0.18; H_3BO_3, 0.1; and $Na_2MoO_4 \cdot 2H_2O$, 0.3. Solid media contained in addition 1% Ionagar no. 2. For submerged cultivations the following procedure was usually adopted. Liquid media (5 ml) in McCartney bottles were inoculated from slopes and incubated as for slopes until they developed (1 to 2 days). These suspensions were used to inoculate 100 ml of medium in 1-liter Erlenmeyer Clearfit (Quickfit and Quartz Ltd., Stone, England) flasks, and a 20% methane in air atmosphere was supplied. Cultures were incubated with shaking at 30 to 33 C for 1 to 2 days and used to inoculate 1 liter of the same medium in 4-liter Erlenmeyer flasks. Finally the 1-liter culture was aseptically transferred to 9 liters of medium contained in 14-liter New Brunswick Microferm fermenters stirred by magnetic transmission. This culture was sparged with a methane-air-CO_2 mixture (100:800:20) ml/min and stirred initially at 150 rpm and as the culture developed this was increased to 400 rpm at 39 to 41 C. Cultures were harvested during exponential growth (Klett reading of 100 to 200 with a red filter) at room temperature, using an air-turbine Sharples Laboratory centrifuge at 50,000 $\times$ *g*. Cell suspensions were not usually washed, but were either disintegrated immediately after resuspension in approximately 10 volumes of 20 mM KH_2PO_4-NaOH buffer (pH 7.1) after cooling to 0 to 4 C or stored as pellets on ice for up to 72 h.

Preparation of cellular fractions. The procedure finally adopted for the separation of different cellular fractions was: the cell suspension at 4 C was disintegrated with a French press and centrifuged sequentially at 3000 $\times$ *g* for 10 min, 10,000 $\times$ *g* for 10 min, 40,000 $\times$ *g* for 20 min, and finally at 109,000 $\times$ *g* for 1 h, at 0 to 4 C. Each pellet was washed twice in the same volume of buffer and resedimented for the same time and at the same centrifugation speed and then finally resuspended in 0.2 to 0.25 volumes of the original buffer and stored on ice. A flow diagram of the procedure is given in Fig. 1.

Electron microscopy of cellular fractions. Fixation, embedding, sectioning, and staining procedures for the particulate fractions were the same as those used previously for whole cells (19, 20). The electron micrographs shown of the sections prepared were obtained with a Phillips EM300 scope (courtesy of H. Haines, Department of Dermatology, University of Miami School of Medicine).

Chemical estimations. Procedures for the estimation of formaldehyde and formate are described in the accompanying paper (24). Carbon dioxide was mea-

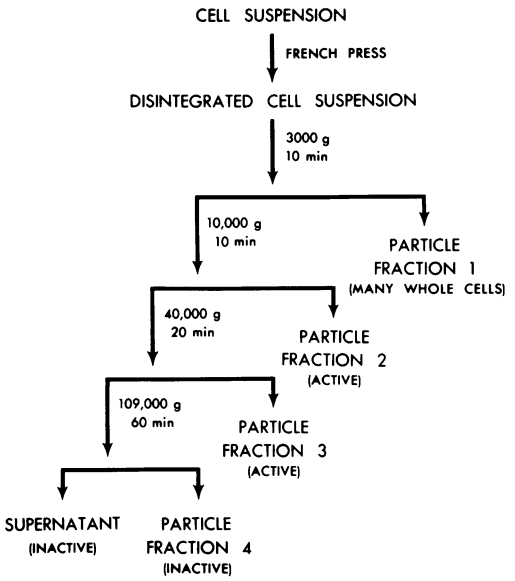

FIG. 1. *Flow diagram of the preparation of cellular fractions of Methylococcus capsulatus.*

sured manometrically by the direct method (23).

Gas chromatography. Dissolved methane and ethane were detected by flame ionization after chromatography in a Carle model 9000 gas chromatograph. Aqueous samples (2 to 5 μl) were directly injected onto a Porapak Q column maintained at 100 C (injection temperature 145 C; detector temperature 200 C.) Helium was the carrier gas flowing at 40 ml/min. Methane and ethane eluted approximately 1 and 3.5 min, respectively, after injection of the samples.

Enzyme assays. NADH consumption was measured spectrophotometrically at 340 nm in a fully automated Unicam SP 800 spectrophotometer. Oxygen consumption was routinely measured polarographically (1) with a Clark oxygen electrode (Yellow Spring Instruments Co., Yellow Springs, Ohio). Oxygen was also measured manometrically in some experiments with constant pressure differential manometers (Gilson Medical Electronics, Madison, Wis.). Spectrophotometric and polarographic measurements do not yield the same absolute values for methane hydroxylase activity because of the sequential oxidations of the products methanol and formaldehyde which are independent of NADH. Simultaneous measurements of oxygen, NADH, and hydrocarbon consumption were made in the cuvette described previously (17) and recorded using three channels of a Rikadenki recorder (Soltec Corp., North Hollywood, Calif.). Methanol oxidase and formaldehyde oxidase activities are recorded as the stimulation of respiration that these substrates allow in the absence of auxilliary electron carriers. Activities are expressed as nanomoles of O_2 consumed per minute. Details of individual experiments appear in the legends to the figures and tables. Dissolved oxygen and methane concentrations at different temperatures were extrapolated from the values given by Chappell (1) and those recorded in *Ullman's Encyklopadie der Technischen Chemie* (22), respectively.

Incubation mixtures described in the tables contained: KH_2PO_4-NaOH buffer (2.8 to 3 ml) saturated with varying proportions of air and hydrocarbons; cofactors and substrates were present as indicated. Conditions of buffer molarity, pH and temperature, and the cellular fractions used are given for individual experiments.

RESULTS

Methane-dependent NADH oxidase activity. Figure 2 shows the progress of oxygen consumption when particles are incubated either alone or in the presence of methane, NADH, or methane plus NADH. Only the latter two incubations significantly stimulate oxygen consumption, and the rate and extent of oxidation are greater in the presence of both methane and NADH than NADH or methane alone. The initial respiratory rates from this experiment were (nmol of O_2 consumed per min): particles alone, 55; particles plus methane, 44; particles plus NADH, 123; particles plus methane plus NADH, 270. Similar relative differences in the rates of NADH disappearance in the presence and absence of methane also occur (18), so that either polarographic or spectrophotometric

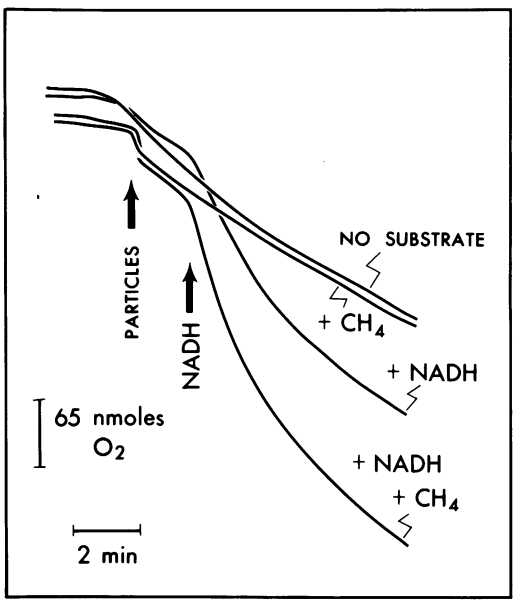

FIG. 2. *Methane-dependent NADH oxidase activity in particulate fractions from M. capsulatus. Reaction mixtures contained: 20 mM KH_2PO_4-NaOH buffer, air saturated (2.7 or 2.5 ml with 0.2 ml of methane-saturated buffer); particle fraction (0.2 ml, containing 1.2 mg of protein); and 25 mM NADH (40 μl) as indicated. Temperature, 30 C.*

measurements are suitable for detecting methane hydroxylase activity.

Distribution of methane-dependent NADH oxidase activity in cell fractions. Methane-dependent NADH oxidase activity resides with the larger particulate matter that sediments between 3,000 and 40,000 $\times$ g. Fractions prepared by centrifugation between 3000 $\times$ g for 10 min and 40,000 $\times$ g for 20 min still contain some whole cells and larger fragments of the cell wall and capsule. Two washes and similar centrifugation gives cell-free preparations. Further fractionation of the particles by two repeated differential sedimentation between 10,000 $\times$ g for 10 min and 40,000 $\times$ g for 20 min yields a preparation containing the larger membrane fragments that is free of whole cells, but cell wall and capsular material are still evident. Fractions obtained between 40,000 $\times$ g and 109,000 $\times$ g contain smaller membrane fragments with no demonstrable methane hydroxylase activity. The various fractions were examined by electron microscopy of thin sections; an example of the 10,000 to 40,000 $\times$ g pellet is shown in Fig. 3. The distribution of NADH oxidase and methane-dependent NADH oxidase activity is given in Table 1. Approximate estimates of the recovery of methane oxidizing activity in cell extracts compared to the unbroken cell suspensions vary between 7 and 15%. This is based on values given in Table 1, for the same preparation, before and after disruption.

Oxidative activities of the particulate fractions. Table 2 shows the effect of various compounds on the respiration catalyzed by particulate fractions. Methane, ethane, propane, and formate alone do not appreciably stimulate respiration. However, methanol, formaldehyde, and dimethylether produce nearly a 20-fold stimulation of the rate of oxygen consumption. Methane or ethane in combination with NADH give similar results whereas the addition of NADH alone results in a fourfold stimulation of respiration. Ethanol or *n*-propanol alone also stimulate respiration by these particles. Formate is not oxidized either in the presence or absence of NADH or NAD.

Effect of extraction medium on the activities of particle fractions. The preparation of particle preparations from *M. capsulatus* with methane-dependent NADH oxidase activity is not reproducible even though the whole cell suspensions that were disintegrated invariably possessed a high respiratory activity when methane or methanol were supplied as substrates. The reasons for this are not clear, and attempts were made to stabilize the activity by modification of the extraction medium. The following additions to 20 mM phosphate buffer (pH 6.8) were made: + 10 mM Mg^{2+}; 15 mM Mg^{2+} plus 1.2 mM spermine; 10 mM Mg^{2+} plus 1 mM adenosine 5'-triphosphate (ATP); 10 mM Mg^{2+} plus 20 mg of bovine serum albumin per ml. Tris(hydroxymethyl)aminomethane-hydrochloride buffer (50 mM) alone or with 10 mM Mg^{2+} plus 1 mM spermine were also tested. The activities extracted with these mixtures from the same batch of cells were not significantly different, with the exception that tris(hydroxymethyl)aminomethane buffer was slightly inhibitory.

Stability of methane-dependent NADH oxidase activity. In spite of the irreproducibility of producing active particulate fractions, those that are active retain activity in 20 mM phosphate (pH 6.8) for days at 0 to 4 C. Stability is not affected by sulphydryl reducing agents, a methane atmosphere, or Mg^{2+} ions.

Effect of particle concentration, phosphate concentration, and pH on methane-dependent NADH oxidase activity. Figure 4 demonstrates the linear relationship between methane-dependent and -independent NADH oxidase activities with the quantity of particles supplied to reaction mixtures.

It has been reported that obligate methylotrophs do not tolerate high phosphate concentrations in growth media (5). Therefore we examined the effect of increasing phosphate concentrations on the methane-dependent and -independent NADH oxidase activities. There is a linear depression of both methane-dependent and -independent NADH oxidase activities when the phosphate concentration is increased from 20 to 680 mM (Fig. 5). The activities at 680 mM are approximately 40% lower than those at 20 mM phosphate, but the stimulation of NADH oxidation by methane is not significantly altered, i.e., 2.1- and 2.25-fold, respectively.

The effect of pH values on the oxidase activities is shown in Fig. 6. There is a decrease of both methane-dependent and -independent NADH oxidase activities as the pH value is raised from 6.0 to 8.0, although between pH values of 6.0 and 7.0 this may not be significant.

Effect of temperature on methane-dependent NADH oxidase activity. Examination of the effect of temperature on the methane-dependent and -independent NADH oxidase activities showed a remarkable dichotomy (Fig. 7). NADH oxidase activity is not appreciably affected by the temperature of incubation between 30 and 57 C. However, the methane-dependent activity is markedly stimulated up to 45 C, when the methane concentration be-

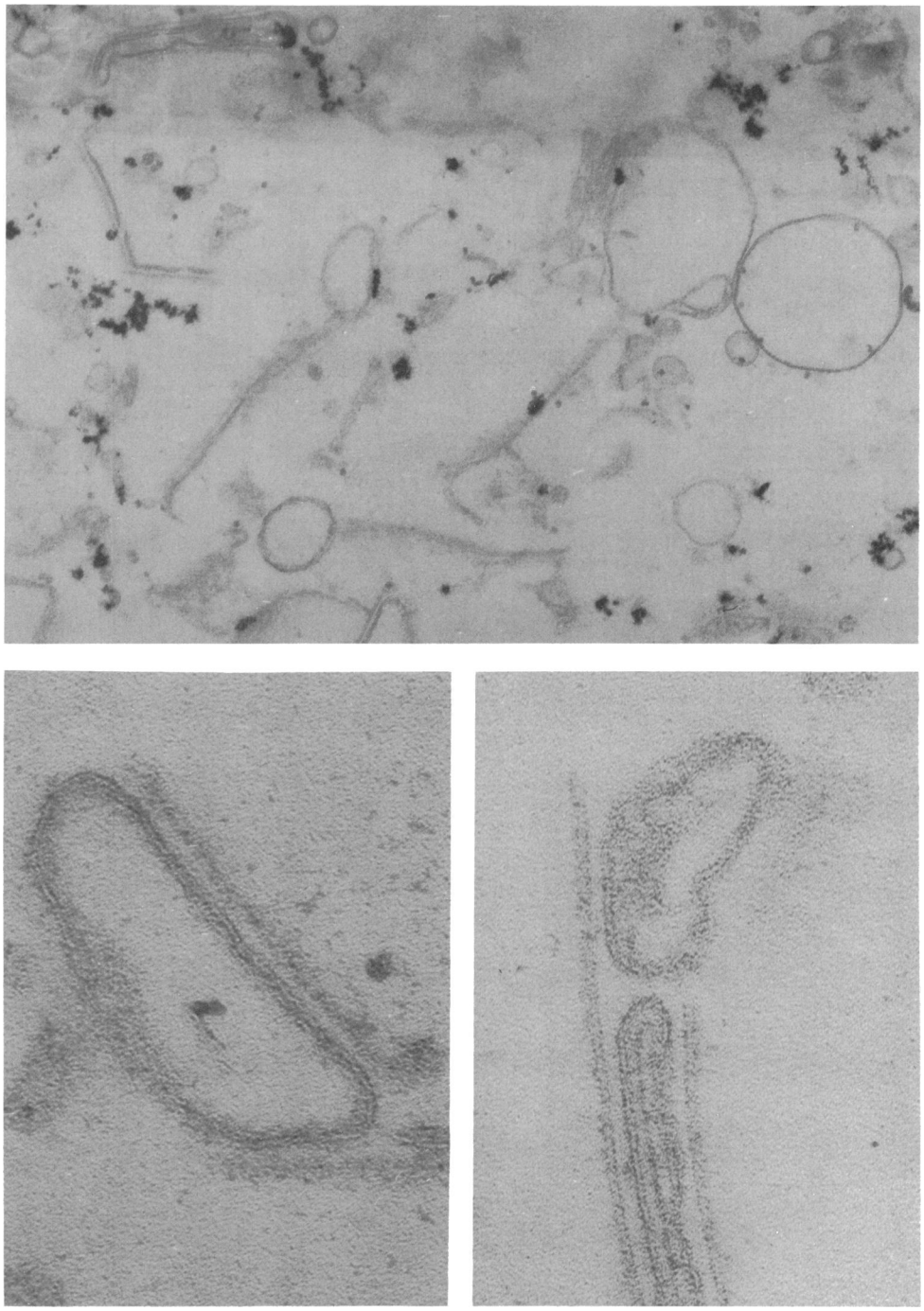

FIG. 3. *Electron micrographs of thin sections of particulate fraction 3. Top: magnification, ×15,000; bottom: magnification, ×60,000.*

comes limiting in the assay. By increasing the methane concentration at the elevated temperatures it can be demonstrated that the methane-dependent activity is stimulated further, up to 57 C, which was the highest temperature used (Fig. 7) in these experiments.

Demonstration of oxygen, NADH, and hydrocarbon consumption. All previous experi-

TABLE 1. *Distribution of methane-stimulated NADH oxidase activity*[a]

Cell fraction	Oxidation rates (nmol of O_2 consumed/min)		
	Endogenous	+NADH	+NADH +methane
Washed cell suspension	25	ND[b]	564[c]
Disintegrated cell suspension	ND	27	48
Particulate fraction1	2 (2.5)[c]	8	30
Particulate fraction 2	ND	18	64
Particulate fraction 3	ND	20	58
Particulate fraction 4	ND	41	45
Supernatant	ND	13	15

[a] Reaction mixtures contained: 20 mM KH_2PO_4-NaOH buffer, pH 6.8, air-saturated (2.8 ml) or 2.5 ml plus 0.3 ml of methane-saturated buffer; cell fraction (0.2 ml); 25 mM NADH (30 μl). Temperature, 37 C. Each particulate fraction was resuspended in buffer to give the original volume of the cell suspension.

[b] ND, Not determined.

[c] Activity in the absence of NADH but the presence of methane.

TABLE 2. *Oxidative activities of a particle fraction from methylococcus capsulatus*[a]

Substrate	Oxygen consumption (nmol/min/mg of protein)
None	10
NADH	45
Methane	14
Methane + NADH	140
Methanol	190
Formaldehyde	120
Formate	16
Formate + NAD	12
Dimethylether	182
Ethane	12
Ethane + NADH	90
Ethanol	75
Propane	12
Propane + NADH	48
n-Propanol	72

[a] Reaction mixtures contained: 20 mM KH_2PO_4-NaOH buffer, air saturated (2.7 ml); particle fraction 2 (100 μl containing 1.4 mg of protein); and 25 mM NADH (30 μl) as indicated. Hydrocarbons were added as saturated solutions in the buffer (0.3 ml). For alcohol, aldehyde, and acid substrates additions of 100 mM solutions (0.2 ml) were made. Temperature, 40 C.

ments reported here and earlier (18) have used indirect assays for measuring hydrocarbon oxidation, i.e., oxygen or NADH consumption

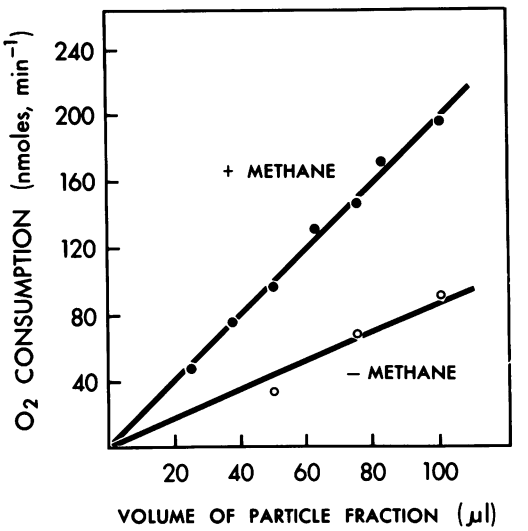

FIG. 4. *Effect of particle concentration on methane-stimulated NADH oxidase activity. Reaction mixtures contained: 20 mM KH_2PO_4-NaOH buffer (pH 7.2), air saturated (2.5 ml); the same buffer, methane saturated (0.5 ml); particle fraction containing approximately 14 mg of protein/ml (25 to 100 μl); reactions were initiated with 25 mM NADH (30 μl). Temperature, 40 C. NADH oxidase activity (O) was measured in 3 ml of air-saturated buffer.*

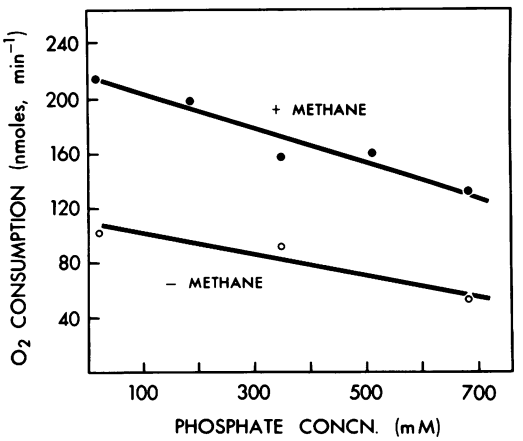

FIG. 5. *Effect of phosphate concentration on methane-stimulated NADH oxidase activity. Reaction mixtures contained: KH_2PO_4-NaOH buffer (pH 7.1), air saturated (2.5 ml); methane-saturated (0.5 ml); particle fraction (100 μl, containing 1.4 mg of protein); 25 mM NADH (30 μl). Temperature, 40 C. NADH oxidase activity (O) was measured in 3 ml of buffer.*

dependent on the presence of hydrocarbon. The two experiments recorded in Fig. 8 and 9 directly demonstrate the consumption of methane and ethane, catalyzed by particle fractions, and this is dependent on the presence of NADH and oxygen. They show the changes in concen-

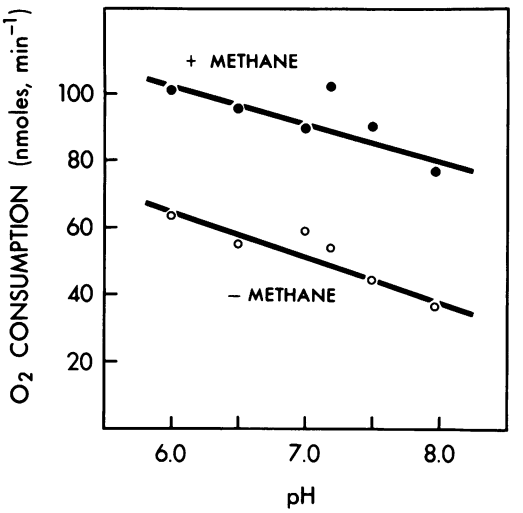

FIG. 6. *Effect of pH on methane-stimulated NADH oxidase activity. Reaction mixtures contained: 20 mM KH_2PO_4-NaOH, air saturated (2.5 ml); methane saturated (0.5 ml), pH as indicated; particle fraction (100 µl containing 1.4 mg of protein); 25 mM NADH (30 µl). Temperature, 40 C. NADH oxidase activity (O) was measured in 3 ml of air-saturated buffer.*

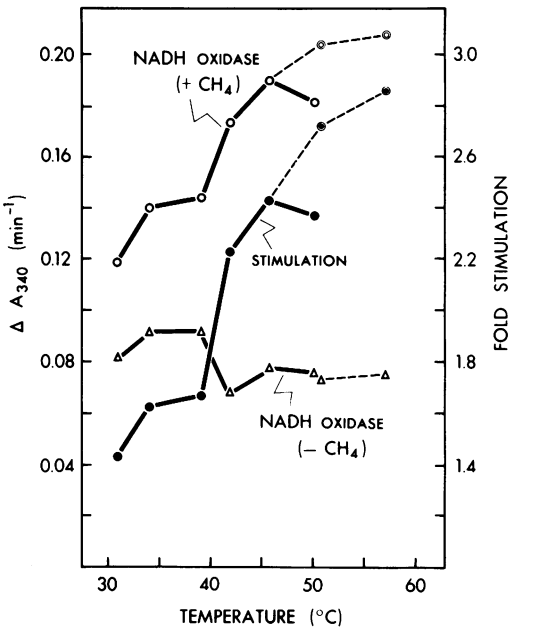

FIG. 7. *Effect of temperature on methane-stimulated NADH oxidase activity. Reaction mixtures contained: 20 mM KH_2PO_4-NaOH buffer, pH 7.1, air saturated (2.8 ml for NADH oxidase activity, 2.5 or 2.0 ml for methane-stimulated NADH oxidase activity); the same buffer, methane-saturated (0.3 or 0.8 ml for methane-stimulated NADH oxidase activity); 25 mM NADH (30 µl); and particle fraction (0.2 ml containing approximately 2 mg of protein). Assays became methane limited at higher temperatures. Higher methane concentrations were employed at 51 to 57 C and are indicated by the double symbols and broken lines.*

trations of hydrocarbon, oxygen, and NADH from single reaction mixtures. It is seen that methane and ethane consumption are dependent on both the presence of NADH and oxygen in the reaction mixtures, since essentially constant values of dissolved hydrocarbon are observed when either NADH (prereaction) or oxygen (postreaction) are absent. These reaction mixtures were limited by oxygen concentration. The reason for the low residual NADH consumption after oxygen depletion is not clear but may represent the NADH oxidase activity (hydrocarbon independent) that could possibly occur by the slow diffusion of air into the cuvette. This may indicate a lower K_m value for oxygen for the NADH oxidase activity relative to the hydrocarbon-dependent NADH oxidase activity.

Formation of formate as a product of methane oxidation. Attempts to determine the stoichiometry of methane and NADH oxidation were inconclusive. Formate was detected as a product of methane oxidation, but the recovery was only about 60% of the expected yield (Table 3). When formate was incubated with the particles and NADH for similar periods, only 60 to 65% of the compound could be recovered. However, qualitatively it is clear that formate is formed as a product of methane oxidation by these particulate fractions.

Requirements for the methane and ethane stimulation of respiration. Both oxygen and NADH are specifically required for hydrocarbon oxidation (Table 4). Electron donors such as NADPH, methanol, and formaldehyde or electron acceptors, NO_3^-, ferricyanide, phenazine methosulphate, or 2,6-dichlorophenolindophenol blue, are not suitable alternatives. The potential role of methanol and formaldehyde as electron donors for methane oxidation cannot be determined from these experiments, since they are also rapidly oxidized by these particle preparations. However, in vivo experiments with washed cell suspensions showed that addition of catalytic quantities of methanol markedly relieves the lag phase of oxygen consumption upon the addition of methane (1), and more effectively than succinate or pyruvate.

Effect of inhibitors on the methane-dependent and -independent NADH oxidase activities. Attempts selectively to inhibit methane-independent NADH oxidase activity but not methane-dependent NADH oxidase activity

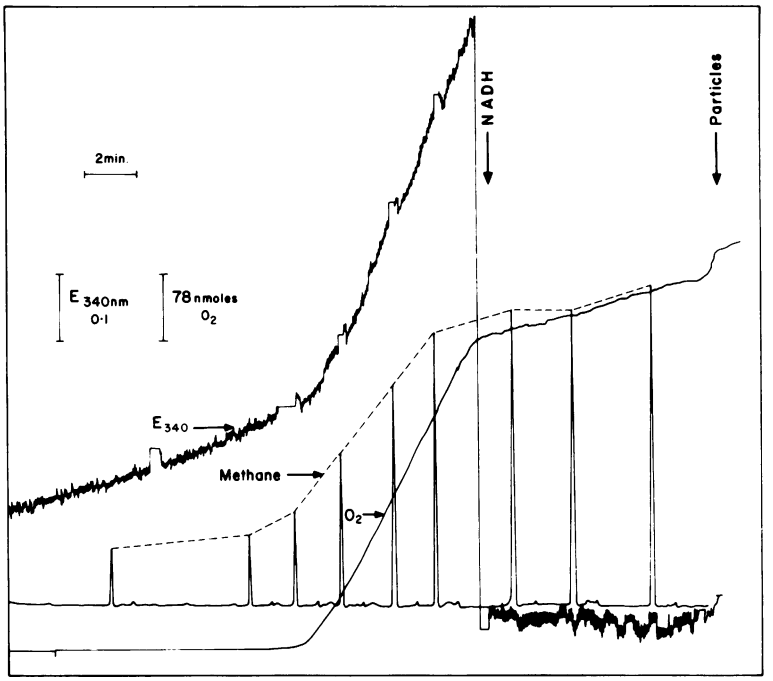

FIG. 8. *Simultaneous assay of methane, oxygen, and NADH consumption catalyzed by particles from M. capsulatus. The reaction mixture contained: 20 mM KH_2PO_4-NaOH buffer (pH 7.2), air saturated (2.0 ml); methane saturated (0.8 ml); and particle fraction (0.2 ml containing 1.8 mg of protein); and reactions were initiated with 25 mM NADH (30 μl). Temperature, 39 C. Samples (5 μl) were withdrawn at intervals from the cuvette and injected directly into the gas chromatograph for methane measurements which were recorded on the same chart as the O_2 and NADH traces. The elution profiles appears 1 minute after the samples were injected. Reaction progress is from right to left.*

with standard inhibitors of electron transport met with no success. Table 5 shows the results obtained. Methane-dependent NADH oxidase activity was very sensitive to azide, carbon monoxide, British anti-Lewisite, hydroxyquinoline *N*-oxide, antimycin A, *o*-phenanthroline, 8-hydroxyquinoline, and acriflavin, but the methane-independent activity was not affected. Cyanide was the only inhibitor found for the latter activity, and this also inhibited the methane-dependent NADH oxidase. The insensitivity of methane-independent NADH oxidase activity to usual inhibitors of electron transport is noteworthy in view of the obligate methylotrophic nature of *M. capsulatus*. This is not shared by the methane-dependent activity. These experiments suggest that electron transport systems of methane-dependent and -independent NADH oxidation are different. It is not clear, however, from parallel experiments conducted for methanol (and formaldehyde) oxidase activities that electrons are transferred via the same carriers to oxygen as those used for NADH (A. M. Wadzinski, Ph.D. thesis, University of Miami, 1973).

DISCUSSION

Differential centrifugation of broken cell suspensions of *M. capsulatus* has yielded cell-free particulate fractions that catalyze the oxidation of methane, methanol, and formaldehyde to formate. Only methane oxidation showed a requirement for NADH. The activity of "methane hydroxylase," which was routinely assayed by the methane-dependent stimulation of O_2 or NADH consumption, was quite variable and frequently very little stimulation was demonstrable. These apparent inactive preparations were not studied further, and only preparations giving at least a two- to threefold stimulation of respiration were used. The reasons for our failure to reproducibly provide active fractions are not at all clear. Slight differences in the physiological state of the cell suspensions, or disintegration conditions, may disrupt the coupled effector (hydrocarbon)-dependent flow of electrons from NADH to oxygen; alternatively, preparations with higher NADH oxidase activities may have been obtained. If the latter were the case then methane-dependent stimulation

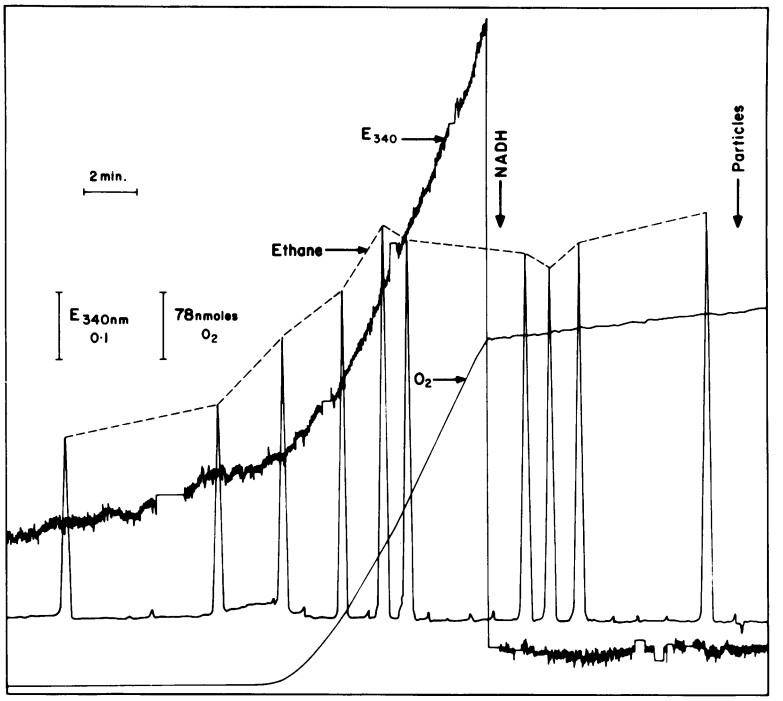

FIG. 9. *Simultaneous assay of ethane, oxygen, and NADH consumption catalyzed by particles from M. capsulatus. Experimental conditions are the same as in the legend to Fig. 8, except that ethane-saturated buffer (0.8 ml) was used. The elution profiles for ethane appear 3.5 min after the samples were injected. Reaction progress is from right to left.*

TABLE 3. *Formation of formate from methane*[a]

Substrates supplied		Substrates consumed		Products formed	
CH_4	NADH	NADH	O_2	Formate	CO_2
0	750	739	322	ND	ND
0	375	370	157	ND	ND
0[b]	10,000	ND	4,700	ND	500
Excess	375	368	239	ND	ND
Excess	250	ND	165	ND	ND
Excess[b]	5,000	ND	11,400[c]	3,200	320
Excess[b]	10,000	ND	24,000[c]	6,800	470

[a] CH_4 (excess) means CH_4 concentration was in excess to NADH concentration on a molar basis. P_2 fraction (1.9 mg of protein) was used for all determinations. All values are expressed as nanomoles. ND, Not determined.

[b] Manometric determinations.

[c] Total gas consumption (O_2 + CH_4).

of NADH oxidation may simply be masked in the routine assays used, and the hydroxylase system may still have been intact. Direct assays of the NADH-dependent consumption of methane (Fig. 8) were not made with such preparations that showed only a small methane-stimulated NADH oxidation. Uncoupling of the electron transport system for NADH oxidation in the methane-dependent pathway, which is distinct from the NADH oxidase pathway, as shown by inhibitors (Table 5) would probably result in the formation of hydrogen peroxide, as shown for a number of other hydroxylases; however, hydrogen peroxide was not detected as a product of oxygen reduction by the catalase assay (unpublished observations). We have tried a variety of disintegration procedures (French press, Hughes press, sonic treatment, grinding, and high-speed shaking with glass beads) and extraction media, but no differences were observed in the activities obtained when applied to the same cell suspension.

The methane hydroxylase activity as assayed routinely, resides in particulate fractions obtained between 3,000 and 40,000 $\times$ *g*. Thin sections of fixed and stained particulate fractions revealed no whole cells in the 10,000 to 40,000 $\times$ *g* pellets; instead the major components were cell wall, capsular, and membranous materials, the latter frequently being vesicular. Fractions separating between 40,000 and 100,000 $\times$ *g* contain only small membrane vesicles, and these did not show appreciable

TABLE 4. *Effect of electron donors and acceptors on the oxidative activities of a particulate preparation*[a]

Substrate	Electron donor	Electron acceptor	Reaction rate (nmol/min/mg of protein)
None	None	O_2	5
	NADH	O_2	60
	NADPH	O_2	8
	NADH	None	4[b]
	Methanol	O_2	149
	NADH	2,6-DCPIP	30[b]
Methane	None	O_2	8
	NADH	O_2	125
	NADPH	O_2	12
	Methanol	O_2	153
	NADH	2,6-DCPIP	24[b]
Ethane	None	O_2	9
	NADH	O_2	95
	NADPH	O_2	12
	NADH	None	4[b]
Methanol	None	O_2	129
	None	PMS + O_2	192

[a] Reaction mixtures and conditions were similar to those described in the footnote to Table 2. 2,6-DCPIP (2,6-dichlorophenolindophenol blue) and PMS (phenazine methosulphate) were added at 800 and 330 μM, respectively; N_2-saturated buffer was used for these experiments. Protein concentration was 1.9 mg/3 ml for all incubations.

[b] Spectrophotometric assay for NADH consumption at 340 nm under anaerobic conditions.

methane-dependent NADH oxidase activity (Table 1).

Methane hydroxylase activity was linearly dependent on particle concentration (Fig. 4), and a distinct pH optimum was not observed between pH values of 6 and 9 in phosphate buffer. The latter observation may be due to the assay used since it probably measures the sequential oxidations of the presumed products of methane oxidation, namely methanol and formaldehyde, and high pH optima are observed for the latter two reactions (12, 24). The effect of phosphate concentration on the methane-dependent NADH oxidase activity was determined to see if the sensitivity of *M. capsulatus* to phosphate concentrations greater than 30 mM in growth media (5) could be attributed to the inhibition of oxidative reactions apparently obligatory for growth on methane. The data in Fig. 5 do not support this notion. Even at 680 mM phosphate the activity is 60% of that at 20 mM, and growth is completely inhibited at 56 mM phosphate (5).

The effect of elevated temperatures on the methane-stimulated NADH oxidase activity is, however, quite dramatic. Between 30 and 57 C there is an almost linear increase in the methane-stimulated activity as the temperature is raised (from 1.4 to 2.9 times the NADH oxidase activity in the experiment reported). Methane-independent NADH oxidase activity remains fairly constant throughout this temperature range. Although this data may be fortuitous, *M. capsulatus* is a thermotolerant bacterium capa-

TABLE 5. *Effect of electron transport inhibitors on the methane-dependent and -independent NADH oxidase activities*[a]

Inhibitor	Concn (mM)	Anticipated site of action[b]	NADH oxidase (% inhibition)	
			CH_4 dependent	CH_4 independent
Azide	110	Terminal	100	0
Cyanide	1.0	Cytochrome	100	77
Carbon monoxide	—[c]	Oxidases	44	Stim
BAL	0.1	Cytochrome *b*	86	0
Urethane	0.1		30	0
HOQNO	0.06	FP → cyt *b* Cyt *b* → cyt *c*	85	0
Antimycin A	0.03	Cyt *b* → cyt *c*	65	0
o-Phenanthroline	0.5	FP → cyt *b*	60	0
8-Hydroxyquinoline	0.1		100	0
Amytal	0.05	NADH → FP	0	0
Acriflavin	0.2	FP	100	0

[a] Reaction mixtures and conditions were similar to those described in the footnote to Table 2. BAL, British anti-Lewisite; HOQNO, hydroxyquinoline; FP, flavoprotein; cyt, cytochrome; stim, stimulation of respiration.

[b] See reference 6.

[c] A one-tenth volume of a saturated solution was used.

ble of growth at 55 C (4), and the ability of the hydroxylase system to function satisfactorily at higher temperatures is therefore obligatory. The greater activities observed at higher temperatures may be an evolutionary consequence, since the availability of dissolved methane will necessarily be lower at higher temperatures. The oxidation of methanol and formaldehyde, also catalyzed by these particles, show similar temperature-dependent profiles (24).

From the limited range of electron donors and acceptors tested (Table 3), methane (and ethane) oxidation show a specific requirement for both NADH and oxygen. This conclusion is supported by direct assays of hydrocarbon consumption (Fig. 8 and 9), which also provide unequivocal evidence that the hydrocarbon-stimulated respiration is coupled to hydrocarbon consumption, and that methane and ethane are not acting simply as effectors or uncouplers of electron flow from NADH to oxygen. The product of methane oxidation by these particles is formate, although it was not correlated with the amount of methane oxidized. The oxidative capabilities of these particles is then consistent with three sequential two electron oxidations of methane linked to oxygen reduction, and the following reaction sequence, which was proposed from whole cell experiments with *M. capsulatus* and other obligate methylotrophs (reviewed in references 15 and 16):

$$CH_4 \rightarrow\rightarrow CH_3OH \rightarrow\rightarrow HCHO \rightarrow\rightarrow HCOOH$$

Our data do not permit the calculation of the stoichiometric relationships for the conversion of methane to formate, although the quantitative conversion of methanol (and formaldehyde) to formate occurs with equimolar (and semimolar) consumption of oxygen respectively (24). The inability to assess the contribution that methane-independent NADH oxidase makes to the consumption of reactants in the presence of methane precludes this determination for the methane to formate conversion. From data presented earlier (18) we had concluded that the equimolar consumption of NADH and oxygen observed in the presence of methane was indicative of a mono-oxygenase reaction. This relationship is not however consistent with the formation of formate which demands the stoichiometry:

$$CH_4 + NADH + H^+ + 2O_2 \rightarrow\rightarrow HCOOH + 2H_2O + NAD^+$$

We have since measured the ratio of NADH:O_2 consumption in several preparations and find that this varies between 1:0.7 and 1:1.3. This large variation might be attributed to the relative amounts of the methane-dependent and -independent NADH oxidase activities in the preparation. The original suggestion was reached on probably fortuitous data. However, the data obtained by Ferenci (3) for methane oxidation by particles from *P. methanica* indicate that NADH and O_2 are consumed in equimolar proportions. It is possible that methanol accumulated as a product in these experiments, accounting for the stoichiometry. The relative rates of methane-dependent and -independent NADH oxidation in these experiments, like those described here, are unknown, and interpretation of the results is equivocal.

The availability of cell-free particulate fractions from *M. capsulatus* containing the intracytoplasmic membranes of the cell, and possessing methane hydroxylase, methanol oxidase, and formaldehyde oxidase activities, allows the tentative conclusion that the extensive intracytoplasmic membrane systems found in this, and possibly other (3), obligate methylotrophs are responsible for these C_1 oxidations in vivo. There is, however, a dearth of knowledge concerning the paths of electron flow from NADH, methanol, and formaldehyde to oxygen, and the potential coupling that may occur to generate ATP. Our attempts to demonstrate respiratory control of methane-dependent or -independent NADH oxidase activity with adenosine 5'-diphosphate and inorganic phosphate have been unsuccessful. Similarly ATP synthesis from adenosine 5'-diphosphate and inorganic phosphate could not be shown. There is yet no information available on the mechanisms of ATP synthesis in obligate methylotrophs, though we have speculated that specialized modes of electron transport coupled to phosphorylation may be the explanation of obligate methylotrophy (16). Thermodynamic calculations of methane oxidation by a reduced nicotinamide nucleotide-dependent oxygenase showed that about 40% of the available free energy change associated with the complete combustion of methane to carbon dioxide would be biologically unavailable unless ATP synthesis was coupled to this oxygenative reaction (16). The fact that methanol and formaldehyde oxidation are probably oxidized by a single dehydrogenase associated with the membranes (24), and that therefore electron transport in vivo is likely to occur by the same route, makes this path of electron flow to oxygen an obligatory scene for ATP synthesis. Clearly ATP synthesis coupled to methane oxygenation is not an essential peculiarity of obligate methylotrophs, since some of these bacteria grow on

methanol (though poorly) as sole source of carbon and energy. The final oxidative step in methane oxidation is the conversion of formate to carbon dioxide. This reaction in *M. capsulatus* (13, 15, 16, 24) and other methylotrophs (15, 16) occurs with the reduction of NAD. NADH is the usual fuel for ATP synthesis in most aerobic biological systems, yet many organic compounds that are oxidized by NAD-linked systems in obligate methylotrophs are unable to support growth, e.g., glucose, pyruvate, and formate. Thus it does not seem likely that NADH oxidation is coupled to ATP synthesis in these organisms as a major source of energy. However, this may be a minor route for ATP synthesis available for maintenance of cells during periods of methane starvation but quantitatively insufficient for growth.

The experiments reported here are in agreement with the original proposal made by Leadbetter and Foster (11) and substantiated by Higgins and Quayle (7) that the initial reaction responsible for the oxidation of methane by methane oxidizing bacteria is catalyzed by a monooxygenase. The data also indicate a major role played by the intracytoplasmic membranes observed in this bacterium (20)—which may account for between 40 and 60% of the total cell mass of these organisms. A similar conclusion might be drawn for *P. methanica* since methane hydroxylase (3) and methanol oxidase (T. Ferenci, personal communication) activities are particulate bound. Our studies with *P. methanica* show that intracytoplasmic membranes, like those in *M. capsulatus*, contain more than half of the cytoplasmic space (unpublished observations; 2). Earlier studies of methane oxidation have been confined to experiments with intact cell suspensions, and the presence of a methane hydroxylase activity has only been inferred from indirect assays of oxygen or NADH consumption catalyzed by particulate fractions from *M. capsulatus* and *P. methanica* (3). However, the reasons for the irreproducibility of this activity in extracts are not apparent. Variation of extraction media for the preparation of these fractions does not seem to affect the activities finally obtained, unlike the specific extraction conditions reported for the "NH_4^+(NH_3) oxidase" activities derived from *Nitrosocystis oceanus* and *Nitrosomonas europea* by Watson et al. (25) and Suzuki and Kwok (21), respectively.

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