METHANE OXIDATION BY CELL-FREE EXTRACTS OF *METHYLOCOCCUS CAPSULATUS*

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1. Introduction

Methane is oxidized to carbon dioxide by several strains of bacteria via methanol, formaldehyde, and formate. Enzymes catalyzing the dehydrogenation of methanol, formaldehyde, and formate have all been demonstrated in extracts of *Pseudomonas methanica, Methanomonas methanooxidans,* or *Methylococcus capsulatus* (for review see [1]). These three species are all obligately dependent upon methane (or methanol) for growth; however, methane oxidation has not been demonstrated in cell-free extracts.

Leadbetter and Foster [2] suggested on the basis of ^{18}O incorporation from $^{18}O_2$ into the cellular constituents of *Pseudomonas methanica,* that methane was oxidized by an oxygenase. This has now been firmly established by Higgins and Quayle [3], who have isolated $CH_3{}^{18}OH$ as the product of methane oxidation when suspensions of *Pseudomonas methanica* or *Methanomonas methanooxidans* were allowed to oxidize methane in $^{18}O_2$ enriched atmospheres. So far attempts to obtain cell-free extracts capable of oxidizing methane have been unsuccessful even though whole cells oxidize methane with great facility. We have now obtained cell-free particulate preparations from *Methylococcus capsulatus* that catalyze methane-stimulated respiration and methane-stimulated NADH oxidation with stoicheiometric relationships consistent with a mono-oxygenase mechanism for methane oxidation.

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2. Materials and methods

Methylococcus capsulatus was grown in mineral salts media [4] in 10 l stirred fermenters, at 38–40°. Harvested cells were disintegrated, after suspension in five volumes of 20 mM KH_2PO_4-NaOH – 10 mM $MgSO_4$ solution, pH 7.2, in a French Press. The extracts were sequentially centrifuged at 3000 *g* for 10 min, 40,000 *g* for 15 min, 40,000 *g* for 10 min, and 100,000 *g* for 60 min. Oxygen consumption was measured polarographically with a Clark oxygen electrode. Spectrophotometric measurements were made in a fully automated Unicam SP 800. Simultaneous measurements of oxygen and absorbance were made in the cuvette described previously [5]. Details of individual experiments appear in the legends.

3. Results

Fig. 1 shows the polarographic assay of methane oxidation by washed suspensions of *M. capsulatus.* This activity is stable for several days at room temperature even in the absence of methane but is completely lost upon freezing or after one passage through the French Press (fig. 1). The crude extract from the French Press, however, exhibited a low methane-stimulated NADH oxidase activity (fig. 2, table 1), which was more pronounced in particle fractions. Methane-stimulated oxidation of NADH could also be demonstrated spectrophotometrically (fig. 2). The methane-stimulated oxidation of NADH resided

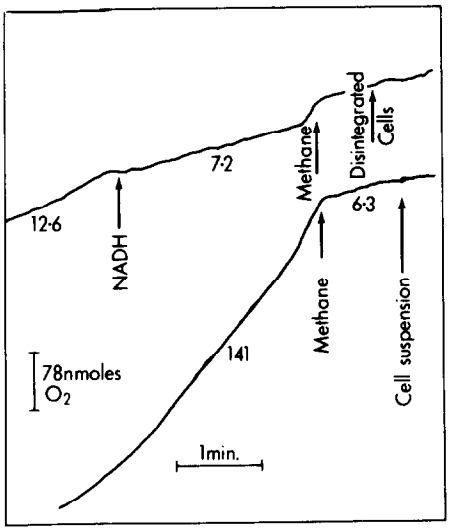

Fig. 1. Polarographic measurement of methane oxidation by washed suspensions and disintegrated suspensions of *M. capsulatus*. The reaction chamber contained: 20 mM KH_2PO_4–NaOH buffer, pH 7.2 (2.5 ml); whole cell suspension (50 µl) or the disintegrated cell suspension obtained after one passage through a French Press (50 µl); and methane-saturated buffer (0.2 ml) as indicated; 25 mM NADH (40 µl) was added as indicated. Temperature, 30°. Time sequence, right to left. Figures appearing quote O_2 consumption rates (nmoles O_2/min).

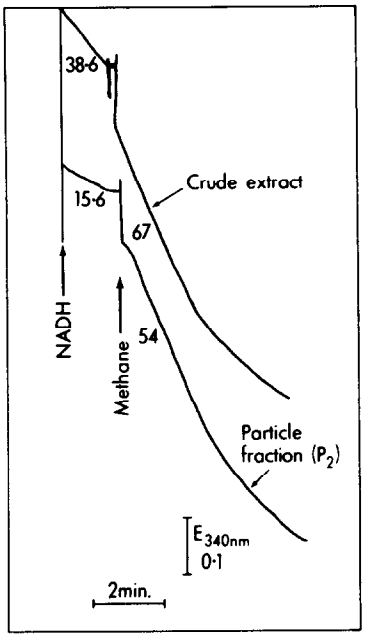

Fig. 2. Spectrophotometric measurements of NADH oxidation in the presence and absence of methane by particle fractions of *M. capsulatus*. Each cuvette contained: 20 mM KH_2PO_4 buffer, pH 7.2 (2.5 ml); and crude extracts or particle fraction P2 (0.1 ml). 25 mM NADH (20 µl) was added to each cuvette and absorbance changes were followed for about 2 min. Methane-saturated buffer (0.3 ml) was then added as indicated. Values under the traces indicate the relative reaction rates (nmoles NADH/min). Temperature, 30°.

mostly in the large particle fraction which sedimented between 3000–40,000 *g*, in 15 min. This fraction contained most of the intracytoplasmic membranes (but fragmented) that are so characteristic of *M. capsulatus* [1, 6, 7], and was free of whole cells but contained some cell wall material. "NADH oxidase" activity is distributed in all sedimentable fractions (table 1).

Simultaneous measurements of oxygen consumption and NADH oxidation are shown in fig. 3. The extent of oxygen and NADH disappearance is proportional with the methane supplied; oxygen and NADH are consumed in equimolar proportions (fig. 4).

Fig. 4. shows the substrate specificity of the mono-oxygenase reaction. Ethane is also readily oxidized, but propane does not appreciably stimulate NADH oxidation. NADPH is not oxidized by these preparations, nor is it an effective electron donor for the alkane-oxygenase reactions in *M. capsulatus*.

4. Discussion

These experiments provide the first demonstration of cell-free oxidation of methane. The soicheiometric relationships for O_2 and NADH consumption further substantiate the conclusion that methane is oxidized by a mono-oxygenase type reaction [1–3]. We have not been able to establish a complete stoicheiometry for the reaction yet, as we have not been able to assess the contribution made by electron flow from NADH to oxygen that may occur independently of methane oxidation. This "NADH oxidase" activity may represent a partial uncoupling of electron transport from the methane oxygenase. However, oxygen and NADH are consumed in the equimolar proportions expected for a mono-oxygenase during hydro-

Table 1
Distribution of methane hydroxylase and "NADH-oxidase" in *Methylococcus capsulatus.*

Cellular fraction	Protein conc. (mg/ml)	NADH oxidase (nmoles/min/ mg protein)	Methane stimulated respiration (nmoles/min/ mg protein)	% Stimulation by methane
Disintegrated cells	14.4	26.8	48.2	42.5
3000 *g* Supernatant	15.0	23.0	33.5	45.8
40,000 *g* Supernatant (15 min)	11.2	14.9	27.5	84.0
40,000 *g* Supernatant (10 min)	9.0	15.9	19.6	23.2
100,000 *g* Supernatant	6.8	13.0	15.0	15.3
3000 *g* Pellet P1	2.2	8.0	30.0	275.0
40,000 *g* Pellet (15 min) P2	5.0	17.7	64.0	262.0
40,000 *g* Pellet (10 min) P2a	3.0	41.0	44.8	9.2
100,000 *g* Pellet P3	4.4	8.6	8.6	0.0

carbon-stimulated respiration (fig. 4). Mono-oxygenases obtained from bacteria have usually been soluble proteins but the methane oxygenase is particulate bound, a not too surprising fact, since bacteria which are able to oxidize methane peculiarly possess extensive intracytoplasmic membrane systems [1, 6–9]. This supports the prediction that these membranes, which appear as well defined stacks in *M. capsulatus* [1, 6, 7], are intimately involved in the oxidation of methane and the transduction of energy from methane.

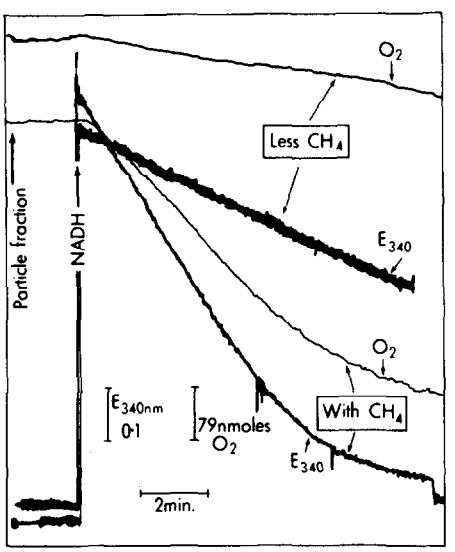

Fig. 3. Simultaneous polarographic and spectrophotometric assay of methane oxidation by particle fraction P2 of *M. capsulatus.* The reaction cuvette contained: 20 mM KH_2PO_4–NaOH buffer, pH 7.2 (2.8 ml ± dissolved methane); particle fraction P2 (0.2 ml); and reactions were initiated with 25 mM NADH (20 µl). Temperature, 30°.

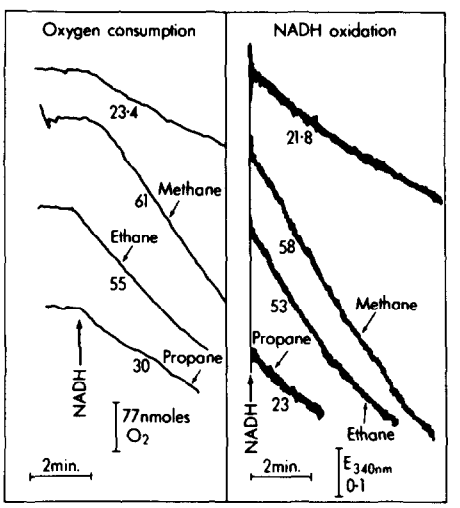

Fig. 4. Substrate specificity of hydrocarbon oxidation by particle fractions of *M. capsulatus.* Simultaneous polarographic and spectrophotometric measurements were made for each reaction mixture but the recorder traces have been redistributed for this presentation. Reaction mixtures contained: 20 mM KH_2PO_4–NaOH buffer, pH 7.2 (2.8 ml ± indicated dissolved hydrocarbon); particle fraction, P2 (0.2 ml); and reactions were initiated by addition of 25 mM NADH (20 µl). Temperature 30°. Figures appearing quote relative reactions rates (nmoles O_2/min and nmoles NADH/ min).

The key reaction of carbon assimilation in *M. capsulatus,* the condensation of formaldehyde with ribose-5-phosphate, is also catalyzed by enzymes extractable from a particulate fraction but it is not yet clear if this activity is an integral part of the membranes or an artefact introduced by disintegration and comminution of the membranes [10]. All pure cultures of methane-oxidizing bacteria, such as *M. capsulatus,* are obligately dependent upon methane as an energy source (methylotrophs) [1] and in this respect resemble autotrophic bacteria such as *Nitrobacter* and *Nitrosocystis* (lithotrophs), which also possess extensive intracytoplasmic membranes. Cell-free oxidation of NH_4^+ ions by extracts of *Nitrobacter europea* and *Nitrosocystis oceanus* have recently been demonstrated and this too is associated with the larger membranous fractions of the cell [11, 12].

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