CARBON MONOXIDE-STIMULATED RESPIRATION IN METHANE-UTILIZING BACTERIA

T. FERENCI

Department of Microbiology, University of Sheffield, Sheffield, S10 2TN, England

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1. Introduction

Methane-utilizing bacteria (methylotrophs) are capable of oxidising a number of one-carbon compounds. Methane is oxidised to carbon dioxide *via* methanol, formaldehyde and formate and these intermediates are themselves oxidised by methylotrophs (reviewed in [1, 2]). Carbon monoxide (CO) has not previously been reported to be an oxidisable substrate of methylotrophs, and the biochemical mechanisms of CO oxidation in the few other bacteria reported to have this capability are poorly understood [2]. It is the purpose of this communication to demonstrate that CO, a compound that does not support the growth of methylotrophs, can however be oxidised by these organisms. Whole-cell suspensions of *Pseudomonas methanica* and *Methylosinus trichosporium* OB3B, members of classes of methylotrophs that differ in their internal membrane structure [3] and their pathways of one-carbon assimilation [4], are both capable of CO-stimulated respiration. Results of work with cell-free extracts of *P. methanica* capable of methane plus NADH-dependent or CO plus NADH-dependent respiration and methane- or CO-dependent NADH oxidation indicate that a mono-oxygenase-type mechanism is involved in both methane and CO oxidation. The properties of the mono-oxygenase responsible for methane oxygenation (methane hydroxylase) in *P. methanica* show many similarities to the activity found by Ribbons and Michalover [5] in extracts of *Methylococcus capsulatus.*

2. Methods

Organisms were grown in 500 ml batch cultures in 2 litre flasks in a mineral salts medium [6] under an atmosphere of methane/air (1:1 v/v). Cells were harvested when the cultures had reached a cell density of 0.06–0.075 mg dry weight of cells. ml^{-1}, and resuspended in buffer containing 20 mM phosphate, 5 mM $MgCl_2$, pH 7.0, to give cell suspensions of 5–10 mg dry weight of cells. ml^{-1}. Cell-free extracts of *P. methanica* were prepared by sonicating cell suspensions at 0–4°C for 90 sec in an MSE 150 W sonicator. The disrupted cell suspensions were centrifuged for 10 min at 6000 *g* to yield the supernatants as the cell-free extracts. Particulate membrane fractions were obtained by centrifugation of the cell-free extracts for 60 min at 38000 *g*.

Formaldehyde was estimated colorimetrically using the method of Nash [7]. The concentrations of saturated solutions of methane and carbon monoxide were taken from solubility tables [8]. Spectrophotometric measurements were carried out in a Unicam SP1800 recording spectrophotometer and oxygen disappearance was followed in a Rank oxygen electrode, both at 30°C.

3. Results and discussion

Initially, cell-free extracts of *P. methanica* were prepared in order to study the properties of the

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Table 1
Methane-stimulated respiration in cell-free extracts of *P. methanica*

Assay	NADH oxidation (nmoles NADH removed.min.$^{-1}$ mg protein^{-1})	Oxygen uptake (nmoles O_2 removed.min.$^{-1}$ mg protein^{-1})
Complete	70.9	50.1
Complete–cell-free extract	0	0
Complete–methane	38.0	15.6
Complete–NADH	0	2.65

NADH oxidation was measured spectrophotometrically by the decrease in extinction at 340 nm, at 30°C. The complete assay mixture, total volume 1.25 ml, in a 1 cm light path cuvette, contained: 50 μmole phosphate buffer, pH 7.0, 0.25 μmole NADH, cell-free extract (containing 0.2–0.5 mg protein, prepared as described in the Methods section) and approx. 0.3 μmole methane, supplied by the addition of methane-saturated water of known methane concentration [8].
Oxygen uptake was followed in an oxygen electrode, at 30°C, using the same quantities of the assay components as for the spectrophotometric measurements above, except higher amount of extract, containing 0.4–1.0 mg protein, were employed.

methane hydroxylase activity and to compare its properties with those observed in *Methylococcus capsulatus* [5]. The procedure described in the Methods section was found to give, reproducibly, active cell-free extracts that exhibited a methane plus NADH-stimulated oxygen uptake (as measured in an oxygen electrode) and a methane-stimulated NADH oxidation (as measured spectrophotometrically), as shown in table 1. After correcting the overall methane-stimulated-rate for methane-independent NADH oxidation and oxygen uptake (i.e. NADH oxidase activity), the methane-dependent rates of oxidation (i.e. methane hydroxylase activity) as shown in table 2. The 1:1 molar ratio observed for methane-dependent NADH disappearance and oxygen uptake is consistent with a stoichiometry that would result from the action of a mono-oxygenase in methane oxygenation.

Many of the properties of the methane hydroxylase in extracts of *P. methanica,* such as the observed stoichiometry of the reactants, the levels of activity found and its location in the particulate fraction of extracts (as indicated in table 2) are similar to those found in extracts of *Methylococcus capsulatus* [5].

Because carbon monoxide (CO) is known to inter-

Table 2
Methane-dependent and carbon monoxide-dependent respiration in cell-free extracts of *P. methanica*

	Methane-dependent:		Carbon monoxide-dependent:	
	NADH oxidation*	Oxygen uptake**	NADH oxidation*	Oxygen uptake**
Cell-free extract	32.9	32.0	32.2	28.1
Particulate fraction	52.0	47.9	66.1	55.0

* nmoles NADH removed.min^{-1} · mg protein^{-1}, after correction for NADH oxidase.
† nmoles oxygen removed.min^{-1} · mg protein^{-1}, after correction for NADH oxidase.
NADH oxidation and oxygen uptake were assayed as described in the legend to table 1. Carbon monoxide-stimulated oxidation was measured in the presence of approx. 0.2 μmole carbon monoxide, supplied by the addition of carbon monoxide-saturated water of known concentration [8] to the assay mixture described in the legend to table 1, instead of methane. The rates shown in the table have been corrected for methane and carbon monoxide-independent respiration (NADH oxidase). The particulate fraction of cell-free extracts was prepared as described in the Methods section.

act with mono-oxygenases associated with cytochrome-P450, which is involved in several bacterial oxygenation reactions [9, 10], the effect of CO was tested on the methane hydroxylase activity. Not only did CO not inhibit methane oxidation, but surprisingly, it was found that CO could itself stimulate NADH oxidation and indeed oxygen uptake. Table 2 shows the rates of CO-dependent NADH oxidation and CO plus NADH-dependent oxygen uptake found in extracts of *P. methanica*, after correction for NADH oxidase activity. The rates of oxidation observed with CO were comparable to those observed with methane as the substrate and the activity was similarly associated with the particulate fraction of cell-free extracts (table 2). The stoichiometry of CO-dependent respiration, in that NADH and oxygen are removed in a 1:1 molar ratio, suggests that CO is also oxidised in a mono-oxygenase-catalysed reaction. Moreover, the enzyme activities responsible for CO and methane oxygenation were both found to be unstable in cell-free extracts and even less stable after separation into the particulate fractions; 80%–90% of both activities were lost on storage of cell-free extracts for 24 hr at 4°C. These similarities suggest a link between the mono-oxygenases responsible for methane and CO oxygenation, possibly through the sharing of a common component in a multi-component system, as was found to be involved in *n*-alkane hydroxylation in *Pseudomonas oleovorans* [11]; at the very least, a common co-substrate (NADH) is used by both mono-oxygenases.

As CO was found to stimulate oxygen uptake in cell-free extracts, the effect of CO on whole-cell respiration was investigated. Table 3 shows the observed rates of oxygen uptake by suspensions of *P. methanica* and *M. trichosporium* OB3B in the presence of the substrates indicated, and the effect of CO.

Resting cell-suspensions of *P. methanica* were found to be unable to oxidise CO, as shown in table 3. This finding can be explained on the basis of the results obtained with cell-free extracts described above, which indicated that CO is likely to be oxidised by a mono-oxygenase. Because CO oxygenation requires a co-substrate in the form of NADH, CO would be expected to be oxidised by whole cells only if an endogenous supply of reducing power were present, and suspensions of *P. methanica*, prepared as described in the Methods section, show little or no endogenous respiration, as indicated in table 3. As also shown, CO inhibited methane-stimulated respiration in *P. methanica;* this can also be explained on the basis of mono-oxygenases being responsible for CO and methane oxygenation, because the presence of CO (which, from preliminary experiments not shown, is preferentially oxidised) is likely to drain endoge-

Table 3
Rates of oxygen uptake by cell suspensions of *P. methanica* and *M. trichosporium* OB3B

Substrate	*P. methanica*		*M. trichosporium* OB3B	
	− CO	+ CO	− CO	+ CO
None (endogenous respiration)	0	0	0	22.9
Methane (0.24 mM)	146	0	91.7	32.1
Methanol (1 mM)	73.8	125	58.9	53.0
Ethanol (1 mM)	31.0	61.8	25.3	22.0
Formaldehyde (3 mM)	47.0	129	81.8	124
Formate (10 mM)	18.9	83.9	27.1	100

Rates of oxygen uptake are expressed as nmole oxygen consumed. min^{-1} · mg dry weight $cells^{-1}$.
Oxygen uptake was followed in an oxygen electrode, at 30°C. In a total volume of 2.5 ml, the reaction vessel contained: 100 μmoles phosphate buffer, pH 7.0, 20–50 μl cell suspension, prepared as described in the Methods section and the substrates at the final concentrations indicated. Rates of oxygen uptake were measured in the absence (− CO) and presence (+ CO) of carbon monoxide, at a final concentration of approx. 0.1 mM.

nous supplies of NADH, which would prevent the oxidation of methane. However, the observed inhibition can also be explained on the basis of a possible inactivation of methane hydroxylase activity by CO. The increase in oxygen uptake observed on the addition of CO to cell suspensions of *P. methanica* oxidising methanol, ethanol, formaldehyde or formate (table 3) is again consistent with the role of a mono-oxygenase in CO oxidation. This is because all these oxidisable substrates may give rise to the intracellular reducing power required for CO oxygenation. The four-fold stimulation of oxygen uptake by CO in the presence of formate (table 3) is especially indicative that CO oxidation can be stimulated by the formation of intracellular reducing power, because the only known enzyme in *P. methanica* that catalyses formate oxidation to CO_2 is an NAD-linked dehydrogenase [12] that would generate NADH in the presence of formate.

Some of the above-mentioned stimulations in oxygen uptake could also be explained on the basis of a CO-stimulated utilization or metabolism of the substrates used in table 3. This possibility was tested by the experiment shown in fig. 1. The figure shows the

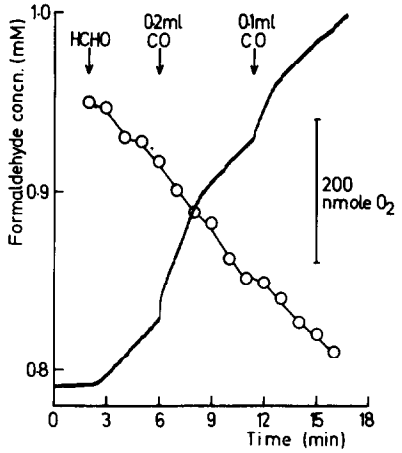

Fig. 1. Oxygen uptake and formaldehyde consumption by *P. methanica*, and the effect of the addition of carbon monoxide. To the reaction vessel of the oxygen electrode containing, in a total of 4 ml, 50 mM phosphate pH 7.0, 0.5 mg dry weight of *P. methanica*, was added, at the times arrowed, 5 μl of 0.75 M formaldehyde and 0.2 ml and 0.1 ml of CO-saturated water (containing approx. 0.86 0.86 μmole/ml CO). Oxygen uptake (continuous trace) was followed and samples were taken for the assay of formaldehyde by the method of Nash [7].

rates of oxygen uptake by a cell suspension of *P. methanica* in the presence of formaldehyde and the stimulation of oxygen uptake upon the addition of limiting amounts of CO. The rate of disappearance of formaldehyde was also followed. The results indicate that the extent of stimulation observed, but not the rate of stimulation, was dependent upon the amount of CO added, indicating CO is not simply an activator of formaldehyde oxidation. Furthermore, in the two additions shown in fig. 1, 108 nmoles and 49.5 nmoles oxygen were consumed upon the addition of 86 nmoles and 43 nmoles CO, again indicating a stoichiometry for the reactants consistent with the involvement of a mono-oxygenase in CO oxidation by *P. methanica*. Also, the 3-fold stimulation in the rate of oxygen uptake upon the addition of CO was not accompanied by a similar increase in the rate of formaldehyde disappearance, again indicating that CO is not just an activator of formaldehyde oxidation but is itself an oxidisable substrate.

The results of experiments with whole-cell suspensions of *M. trichosporium* OB3B showed several differences from those obtained with *P. methanica*. As shown in table 3, CO can be oxidised by suspensions of *M. trichosporium* in the absence of any added carbon source and CO does not stimulate oxygen uptake in the presence of methanol or ethanol. The two methylotrophs are similar in that CO was found to inhibit methane-stimulated oxygen uptake and stimulate formaldehyde or formate-dependent respiration. These results suggest that although methane and CO may both be oxygenated by a mono-oxygenase in *M. trichosporium* OB3B, as in *P. methanica*, there may be some differences between the oxidative pathways of these methylotrophs consistent with differences between their membrane structures [3] and carbon assimilation pathways [4]. Further work is needed to clarify this point.

Growth studies with *P. methanica* and *M. trichosporium* OB3B in liquid cultures have shown that CO was unable to support the growth of either organism when present as a sole carbon source. Growth on methane was totally inhibited by CO, and growth on methanol partially inhibited, in both methylotrophs.

A recent report has shown the interaction of CO with cytochromes found in methylotrophs [13]. Davey and Mitton [13] also suggested an involvement

of a CO-sensitive cytochrome at an early stage in methane, but not methanol, oxidation on the basis of some unpublished work. The data presented in this paper makes it likely that the 'CO-sensitive cytochrome' [13] may be associated with the monooxygenase(s) found in *P. methanica.*

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