PROPERTIES AND PARTIAL PURIFICATION OF THE METHANE-OXIDISING ENZYME SYSTEM FROM *METHYLOSINUS TRICHOSPORIUM*

G. M. TONGE, D. E. F. HARRISON, C. J. KNOWLES and I. J. HIGGINS

Biological Laboratory, University of Kent, Canterbury, Kent, CT2 7NJ, UK and Woodstock Laboratory, Shell Research, Ltd., Sittingbourne, Kent, ME9 8AG, UK

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1. Introduction

The nature of the initial enzymic attack on methane by obligate methylotrophs has attracted considerable research effort in recent years. Strong evidence for oxygenase involvement came from ^{18}O experiments which demonstrated that the oxygen in methanol, excreted by washed suspensions of *Methanomonas methanooxidans* and *Pseudomonas methanica*, incubated under methane + oxygen mixtures was derived exclusively from molecular oxygen [1]. Subsequently, there were reports of NADH-dependent methane oxidation in cell-free preparations from both *Methylococcus capsulatus* [2,3] and *Pseudomonas methanica* [4]. However, detailed studies and purification of these enzyme systems have not proved possible hitherto, due to their particulate nature and apparent instability. The products of cell-free methane oxidation were not identified but the stoichiometries obtained using methane [2,4] and the substrate analogue, carbon monoxide, which is oxidised to carbon dioxide [4] suggest the enzyme is a mono-oxygenase.

The current paper describes similar activity in *Methylosinus trichosporium*. However, in addition to NADH, this system can be linked to other electron donors (especially ascorbate) via a soluble CO-binding *c*-type cytochrome previously reported by us to occur in a variety of methylotrophs [5]. This cytochrome has been highly purified and there is good evidence that it is the natural electron donor for the methane oxygenase enzyme. The immediate product of methane oxidation, methanol, can serve as the electron donor for the oxygenase in crude extracts that contain methanol oxidase activity. This most likely reflects recycling of electrons derived from the methanol dehydrogenase reaction, via the cytochrome to the oxygenase.

A novel assay procedure has been developed in which methanol formation from methane is measured in the presence of high phosphate concentration which inhibits the further oxidation of methanol [1,6]. Use of this assay has revealed that preparations which were apparently inactive or had lost activity on the basis of lack of methane stimulation of NADH-dependent oxygen consumption, were often highly active on the basis of methanol formation. That is, methane addition causes a redirection of electron flow to oxygen rather than any overall stimulation of the rate of oxygen consumption. By using the new assay, it has become clear that the methane oxidising system of *M. trichosporium* is both stable and highly active. The oxygenase component has been solubilised from particle preparations effecting a considerable purification.

2. Materials and methods

Methylosinus trichosporium OB 3b was a gift from Professor R. Whittenbury and was grown on methane supplied as a methane–air mixture, at 30°C in a simple mineral salts medium [7], usually in oxygen-limited continuous culture (dilution rate, 0.06 h^{-1}). Organisms used for cytochrome purification were grown under similar conditions in a 200L batch culture. Harvesting, preparation of cell-free extracts and of 150 000 *g* particle preparations were as described previously [5]

except the particle preparation was resuspended in 20 mM sodium phosphate buffer, pH 7.0 containing 5 mM $MgCl_2$.

Cytochrome spectra and protein concentrations were obtained as described previously [5], routine spectrophotometric measurements were carried out on a Pye-Unicam SP1800 recording spectrophotometer and oxygen consumption followed in a Rank oxygen electrode at 30°C. Methane and methanol concentrations in reaction mixtures were determined gas chromatographically using a Porapak Q glass column (80–100 mesh, 2.1 m × 4 mm i. d. in a Pye 104 gas chromatograph fitted with a flame ionisation detector, using a nitrogen flow rate of 40 ml/min at 125°C.

Except when stated otherwise, methane oxidising activity was measured by following methanol formation. Reaction mixtures contained; methane (0.2–0.3 μmol), NADH (7.8 μmol) or ascorbate (4.5 μmol), pure CO-binding cytochrome *c* from *M. trichosporium* (4.5 nmol), test protein (20–500 μg) and sodium phosphate buffer, pH 7.0 (450 μmol) in a total volume of 3.0 ml incubated at 30°C in a Rank oxygen electrode.

The CO-binding cytochrome *c* was purified by a similar procedure to that described by Anthony for the cytochrome *c* of *Pseudomonas* AM1 (6). Its mol. wt. was estimated by gel filtration [8] and redox potential by redox titration [9,10]. Disc gel electrophoresis was carried out using 10% total acrylamide running gels and 3.3% total acrylamide stacking gels [11]. Polyacrylamide gel electrophoresis of sodium dodecylsulphate-treated proteins was carried out as described in [12].

3. Results

3.1. *Cell-free methane oxidising activity*

Addition of methane-saturated buffer to crude sonicated extracts oxidising NADH (190–200 nmol/min/mg of protein) often did not stimulate oxygen utilisation significantly. Occasionally, methane-dependent oxygen uptake of about 30 nmol/min/mg of protein was recorded but this activity was always lost after storage for 24 h at 4°C. This value is similar to those quoted in extracts of other methane utilisers [2,4].

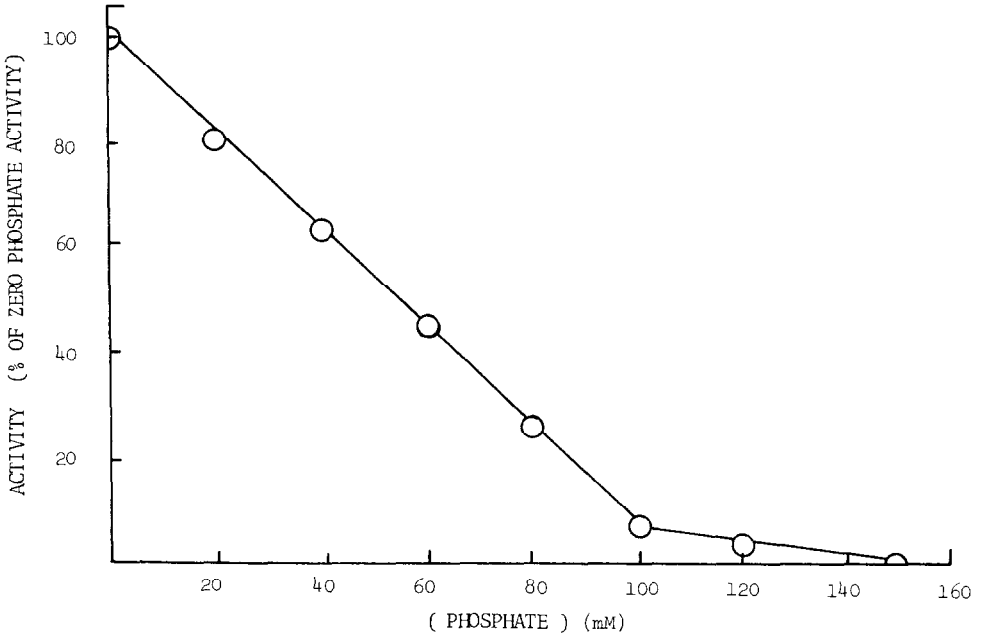


Fig.1. Effect of phosphate concentration on methanol oxidation by a cell-free extract of *M. trichosporium*. Methanol-dependent oxygen consumption was measured polarographically at 30°C in the presence of various concentrations of sodium phosphate buffer, pH 7.0. Reaction mixtures contained in addition to buffer, methanol (16 μmol) and extract protein (1.5 mg) in a total volume of 3.0 ml. The zero phosphate result was obtained using imidazole buffer (50 mM).

Table 1
Methane oxidising activity of cell-free extract and particle preparations from *M. trichosporium*

Preparation	Electron donor	Electron donor concentration (mM)	Phosphate concentration (mM)	Specific activity (nmol/min/mg of protein)
Cell-free	NADH	2.6	20	0–30
extract	NADH	2.6	150	220
	Ascorbate	1.5	150	210
	Methanol	38	20	177
	Methanol	38	150	0
	Formate	5	150	20
	Formate + NAD^+	5 2.6	150	250
+ Amytal (4 mM)	NADH	2.6	150	0
+ Amytal (4 mM)	Ascorbate	1.5	20	0
+ Amytal (4 mM)	Ascorbate	1.5	150	224
150 000 *g* particles	Ascorbate + purified cyt.*c*	1.5	20	0
	Ascorbate + purified cyt.*c*	1.5	150	730

Methane oxidising activity was usually determined by following methanol formation as described in Materials and methods, in the presence of electron donors and inhibitors as tabulated. However, when methanol served as electron donor activity was measured by following methane utilisation gas chromatographically.

Extracts also contained methanol oxidase activity of about 170 nmol/min/mg of protein when measured in 20 mM phosphate buffer. However, this activity could be progressively inhibited by increasing the phosphate concentration in the reaction mixture (fig.1). Complete inhibition occurred at 150 mM phosphate but this concentration had little effect on NADH oxidase activity or methane-dependent oxygen consumption. When 150 mM phosphate was included in incubation mixtures and methanol formation from methane measured at timed intervals, the specific activity was 220 nmol of methanol formed/min/mg of protein (table 1). All extracts showed similar activity when measured in this way including those prepared from washed suspensions of organisms stored at −15°C for 12 months. In addition, there was no significant loss of activity after storage of extracts at 4°C for 14 days. Clearly oxygen consumption rate measurements are not a true reflection of methane oxidising activity.

All methane oxidising activity was located in particulate fractions obtained by centrifugation at 150 000 *g* for 90 min and the specific activity of these was somewhat higher than that in the crude extract (table 1). The methanol oxidase activity of the particles was only 30 nmol/min/mg of protein. Methane oxidising activity was stable on storage of the particles at 4°C provided that purified CO-binding cytochrome *c* from *M. trichosporium* was included in assay mixtures. On preparation of these particles, most of this cytochrome is released into the supernatant fraction [5] and the remainder slowly leaches from the particles on storage. Failure to supply exogenous purified cytochrome resulted in low activities of methane oxidation.

3.2. *Electron donor specificity*

Ascorbate (1.5 mM) was an equally effective electron donor when substituted for NADH both in crude extracts and in particle preparations (table 1). Methanol could also serve as the electron source provided the phosphate concentration was low (20 mM). In crude extracts, the rate of methane utilisation in the presence of methanol was 177 nmol/min/mg of protein, closely similar to the methanol oxidase activity measured polarographically. This suggests that in these experiments the rate of methane oxidation is limited by the rate of methanol oxidation, supporting the contention that methanol itself is serving as the (indirect) electron source for the methane oxygenase.

The possibility that electrons from ascorbate were reducing endogenous NAD^+ by some type of reversed electron transport process was investigated. Amytal (4 mM) totally inhibited NADH oxidase activity in crude cell-free extracts without having any effect on methanol oxidase activity or ascorbate-dependent methane oxidation, but it completely inhibited NADH-dependent methane oxidation (table 1). In addition, if formate was added, which might be expected to serve as an electron donor because of endogenous formate dehydrogenase activity, there was little methane oxidation unless exogenous NAD^+ was supplied. This suggests that the endogenous NAD^+ concentration is not sufficient to permit the observed rates of methane oxidation if this coenzyme is involved in the transfer of reducing power from ascorbate.

3.3. *Properties of the CO-binding c-type cytochrome and its role in methane utilisation*

There is strong evidence that the CO-binding *c*-type cytochrome of several methanol-utilising bacteria is intimately involved in methanol oxidation [5,6,13]. This cytochrome, purified from *M. trichosporium* (single sharp band on both disc and SDS gels after 33-fold purification; two faint diffuse bands on SDS gels only) has similar properties to that obtained from *Pseudomonas* AM1, having a mol. wt of 12 400 (SDS gel electrophoresis) – 12 500 (gel filtration) and a redox potential of + 310 mV but differs in being readily autooxidisable. At all stages during purification, the cytochrome showed a high degree of CO-binding-66-82% (5, 14). Therefore, the CO-binding was not due to traces of denatured cytochrome *c*. The final preparation was greater than 90% pure on the basis of published molar extinction coefficients [14].

During this study it became apparent that there was a close relationship between the concentration of this cytochrome in cell-free preparations and methane oxidising activity. For example, some of the cytochrome remains in 150 000 *g* particles when they are first prepared (about 11% of the total) and this is reflected by residual methanol oxidase activity in these particles (about 17% of the total). Washing with 20 vol of sodium phosphate buffer

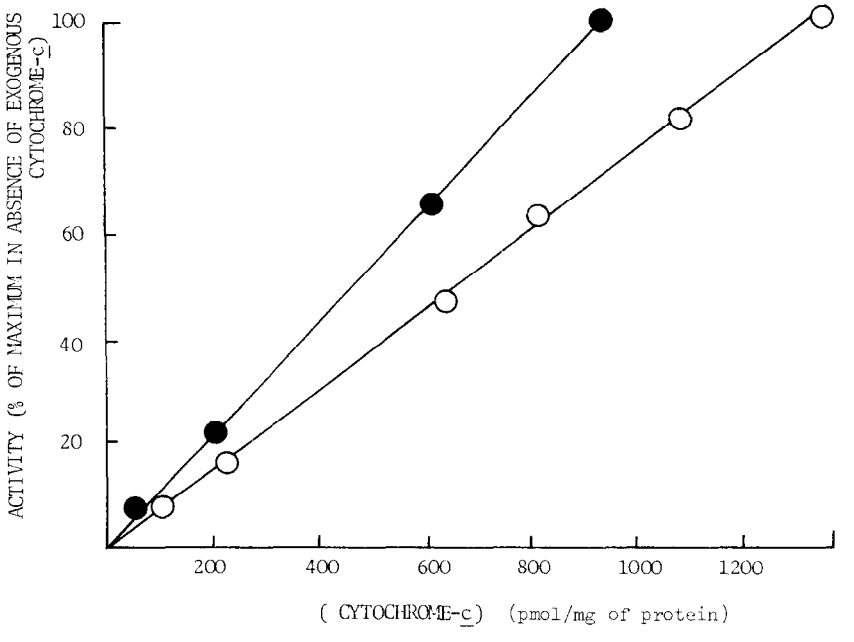


Fig.2. Relationship between endogenous CO-binding *c*-type cytochrome concentration and the rate of methane (○) and methanol (●) oxidation. Particulate preparations containing various residual concentrations of the CO-binding cytochrome-*c* were obtained by centrifugation under different conditions (38 000–200 000 *g* for 60–90 min.) Methanol oxidase activity was measured as described for fig.1, methane oxidising activity and cytochrome concentration as in Materials and methods.

(20 mM, pH 7.0) containing magnesium chloride (5 mM), followed by recentrifugation at 150 000 *g* for 90 min removed the remaining cytochrome and associated methanol oxidase activity from the particles. In addition, methane oxidising activity was lost completely but could be fully restored by adding back the purified CO-binding cytochrome *c* (1.5 μM). Fig.2 shows the relationship between both methane oxidising and methanol oxidase activities of particle preparations and their cytochrome *c* contents when activities were measured in the absence of exogenously-supplied purified cytochrome. The different preparations were obtained by various centrifugation procedures including treatment for 90 min at 150 000 *g*, 100 000 *g* [2] and 38 000 *g* [4].

3.4. *Partial purification and properties of the methane oxygenase system*

Table 2 shows the results of using various techniques to solubilise the oxygenase component from the 150 000 *g* particulate fraction. Treatment with phospholipase-D, Triton X-100 or ultrasound were all effective in releasing activity into the supernatant obtained after recentrifuging at 150 000 *g* for 90 min. Phospholipase is clearly the preferred procedure since, although under these conditions solubilisation was incomplete, there was a large increase in specific activity, representing a 38-fold purification from cell-free extract. Examination of disc gels indicated one major band associated with smaller amounts of several other proteins (including phospholipase) with higher mobilities. Attempts at further purification are currently in progress.

This soluble preparation contains little activity unless the purified CO-binding cytochrome *c* is added, together with either ascorbate or NADH. It is entirely free of endogenous NADH oxidase activity. Using NADH as electron donor, the stoichiometry, methane utilisation:methanol formation:NADH utilisation: oxygen consumption was 1.0: 0.9: 1.6: 1.3. There was no evidence of dimethyl ether formation by this preparation and no loss of activity on storage at 4°C for 7 days. Release from the particles by ultrasonication without loss of activity also suggests a relatively stable enzyme.

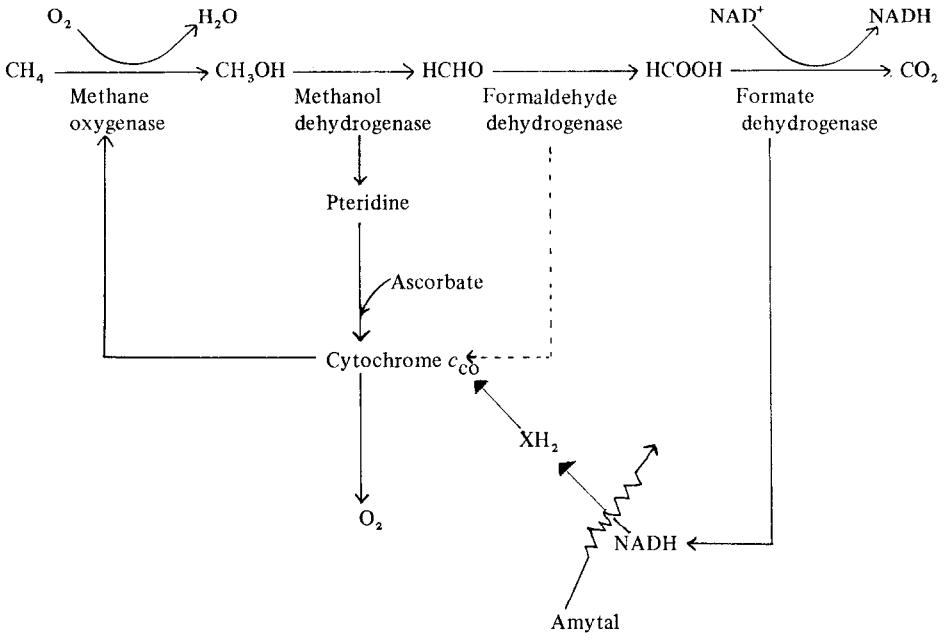
4. Discussion

The results show that the methane oxidising enzyme is located in particulate fractions, most likely derived from the internal membranes of *M. trichosporium*, and that it is stable and can be readily solubilised from these particles. The stoichiometry suggests that it is a monooxygenase, but the figures for NADH and oxygen consumption are slightly high. They probably reflect a portion of the electron flow being direct to oxygen via the CO-binding cytochrome *c*, i.e. conventional NADH oxidase activity. Alternatively the figures would be consistent with some uncoupling of electron flow from oxygenation, i.e. methane-dependent NADH

Table 2
Solubilisation of methane oxygenase from *M. trichosporium* 150 000 *g* particle preparations.

Treatment	Oxygenase specific activity (nmol/min/mg of protein)		Proportion of total activity solubilised (%)	Recovery of activity from original particles (%)
	Supernatant	Particles		
0.1% Triton X-100	1140	550	35.9	nd
1% Triton X-100	1536	372	66.7	99
2% Triton X-100	1880	240	81.7	100
Sonication	1300	0	100	100
Phospholipase-D	5500	640	66.2	100

nd = Not determined.
Samples of a 150 000 *g* particle fraction containing 54 mg of protein/ml were subjected to the following procedures before recentrifuging for 90 min at 150 000 *g*: (a) incubation at 4°C with vigorous shaking for 1 h in the presence of 0.1–2% Triton X-100, (b) ultrasonication for 4 × 30 sec using an MSE Type 150 W sonicator, (c) incubation at 30°C for 1 h with 400 μg/ml of phospholipase-D. Methane oxidizing activity was determined by the standard procedure.



Scheme 1. Tentative mechanism for methane oxidation by *M. trichosporium*

oxidase activity. Such uncoupling has been noted in several other mono-oxygenase preparations, for example, orcinol hydroxylase [15,16]. Associated CO-binding cytochrome *c* seems to be essential for activity. It is not possible to differentiate between these two possibilities at present since the pure CO-binding cytochrome *c* cannot be reduced by NADH in the absence of the partially purified oxygenase component. This may contain another electron carrier (XH_2 in scheme 1) or it is possible that the oxygenase itself facilitates reduction of the cytochrome by NADH.

Our results are consistent with a mechanism for methane oxidation shown in scheme 1, in which electrons derived from methanol (and perhaps formaldehyde) oxidation are recycled into the methane oxygenation reaction. There is evidence that formaldehyde is also a substrate for methanol dehydrogenase [17].

Since, in crude extracts methanol can serve as the electron donor for methane oxygenation and NADH is not involved when ascorbate is the electron donor, the contention of van Dijken and Harder [18] that reversed electron transport is obligatory for growth on methane is clearly not tenable. Recycling of electrons from the oxidation of methanol and perhaps formaldehyde obviates the necessity for reversed electron transport. Further, we propose that the normal physiological electron donor is not NADH, but rather the immediate donor is the CO-binding cytochrome *c*, being reduced in turn by the methanol dehydrogenase.

The $CH_3OH/HCHO$ and HCHO/HCOOH redox couples have values of E_0'(pH 7.0) of −182 and −450 mV respectively [19] and the redox potential of the CO-binding cytochrome *c* is + 310 mV. It is thermodynamically possible therefore that one or two moles of ATP are formed by oxidative phosphorylation per mole of methanol oxidised to formaldehyde. In the scheme suggested here, no extra ATP would be generated from the first oxidation step, methane to methanol. However, one or two molecules of ATP per electron pair could be generated during the reduction of the cytochrome *c*, linked to the methanol and formaldehyde dehydrogenase reactions. In this case, the reducing potential utilised in the first step of methane oxidation may not be at the expense of ATP formation from methanol. Alternatively it is possible that some of the ATP is produced during the oxidation of the cytochrome *c* by oxygen and that this would be lost each time reduced cytochrome *c* was used for methane

oxygenation. Assuming the former case, on a molar basis, the efficiency of growth on methane could equal that on methanol. This would seem to agree with observed results [20] which are difficult to explain in terms of a conventional NADH-linked mono-oxygenase [18].

It is notable that the CO-binding cytochrome *c* of *M. trichosporium* is autooxidisable, unlike a similar cytochrome isolated from *Pseudomonas* AM1 [6]. This is consistent with many preparations not showing any increase in NADH- or ascorbate-dependent oxygen consumption on adding methane. It seems likely that in *M. trichosporium*, the cytochrome functions as an oxidase or donates electrons to oxygen via an oxidase in the absence of methane, but in the presence of the oxygenase component, addition of the hydrocarbon results in a coupling of pre-existing electron flow to methane oxygenation. Our observation that the cytochrome *c* is involved in the methane oxygenase reaction probably explains the CO-binding ability of the cytochrome, which is unusual in non-denatured *c*-type cytochromes [5,14]. A clear analogy exists between this system and the cytochrome *P*-450-containing higher alkane mono-oxygenase found in a *Corynebacterium* [21]. It remains to be determined whether oxygenase functions are common to CO-binding *c*-type cytochromes and it is not yet clear whether the one in *M. trichosporium* is involved in carbon monoxide oxidation demonstrated in methane-utilising bacteria by Ferenci [4].

In view of our findings, low methane oxidising activities and lack of stability reported for other methane-utilising bacteria may be artefactual and due to measuring oxygen consumption rather than product formation. Loss of the CO-binding cytochrome *c* from particle preparations would also account for loss of activity.

This study demonstrates the danger of using indirect assay procedures especially in the study of oxygenases. This problem has been discussed previously [22]. In the current work, measurement of activity in crude extracts by polarography gave at best, rates representing about 10% of the true activity and at worst, no rate whatsoever.

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