Regulation of the Intracellular Location of Methane Mono-oxygenase During Growth of *Methylosinus trichosporium* OB3b on Methanol

By KAREN J. DAVIS,* ALEX CORNISH† AND I. JOHN HIGGINS

Biotechnology Centre, Cranfield Institute of Technology, Cranfield, Bedfordshire MK43 0AL, UK

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The methane-oxidizing bacterium *Methylosinus trichosporium* OB3b retains the capacity to express two types of methane mono-oxygenase (MMO) during growth on methanol in continuous culture. Soluble MMO predominates during copper-limited growth whilst particulate (membrane-bound) MMO activity is expressed fully only in copper-sufficient cultures. Organisms containing soluble MMO oxidized ethylbenzene (an artificial substrate for soluble but not particulate MMO) but this capacity was progressively lost when additional copper was made available to the organism, and MMO activity was transferred to the particulate fraction of cell-free extracts. Although intracellular location of MMO is apparently regulated by copper availability during growth on both methane and methanol, whole-cell MMO activities are appreciably higher during growth on the former (natural) carbon source.

INTRODUCTION

Methanotrophs oxidize methane using the sequence of enzyme-catalysed reactions shown in Fig. 1 (Higgins *et al.*, 1981). The oxidation of methane to methanol is catalysed by methane mono-oxygenase (MMO) systems, of which two distinct types, one soluble and the other particulate (membrane-bound), occur in both *Methylosinus trichosporium* OB3b (Scott *et al.*, 1981; Burrows *et al.*, 1984) and *Methylococcus capsulatus* (Ribbons & Wadzinski, 1976; Stanley *et al.*, 1983) during growth on methane. The soluble MMO oxidizes a wide range of hydrocarbons in addition to methane (e.g. *n*-alkanes, *n*-alkenes, aromatic and alicyclic compounds) and uses only NAD(P)H as an electron donor (Colby *et al.*, 1977; Stirling *et al.*, 1979; Burrows *et al.*, 1984). Particulate MMO, on the other hand, oxidizes only *n*-alkanes and *n*-alkenes (Dalton *et al.*, 1984; Burrows *et al.*, 1984) and functions with NAD(P)H and additional electron donors (Leek & Dalton, 1983; Cornish *et al.*, 1985). Copper availability determines which MMO is present in both *M. trichosporium* OB3b and *Methylococcus capsulatus* (Bath) during growth on methane. Particulate MMO exists when the organisms are grown under conditions of copper sufficiency, whilst soluble MMO exists when they experience copper deficiency (Stanley *et al.*, 1983; Burrows *et al.*, 1984; Dalton *et al.*, 1984).

Some methanotrophs can also grow on methanol, although on this carbon source MMO is theoretically unnecessary for growth (Fig. 1). There have been conflicting reports concerning the levels of MMO activity and the intracellular location of the methane oxidizing system when methanotrophs are grown on methanol instead of methane. Hou *et al.* (1979) reported that several methanol-grown methanotrophs, including *M. trichosporium* OB3b, lost the capacity to oxidize methane. By contrast, Best & Higgins (1981) found that *M. trichosporium* OB3b retained MMO activity during growth on methanol in batch culture and carbon-limited continuous culture and MMO activity was always located in the soluble fraction of cell-free extracts. Prior & Dalton (1985) reported that *Methylococcus capsulatus* (Bath) contained only particulate MMO

† Present address: Department of Biochemistry, University of Leicester, Leicester LE1 7RH, UK.

Abbreviation: MMO, methane mono-oxygenase.

Fig. 1. Generalized scheme for the oxidation of methane by methanotrophs. 1, MMO; 2, methanol dehydrogenase; 3, formaldehyde dehydrogenase; 4, formate dehydrogenase; PQQ, pyrrolo-quinoline quinone.

activity during growth on methanol in carbon-limited continuous culture: soluble MMO was never detected, even when the organism was grown on a copper-free medium, but particulate MMO activity increased significantly when additional copper was made available.

It has been suggested that the 'soluble' MMO activity reported by Best & Higgins (1981) might have been due to particulate MMO released from membranes during sonic disruption of *M. trichosporium* OB3b (Prior & Dalton, 1985). This seems unlikely, since the methanol-grown cultures used by Best & Higgins (1981) had the capacity to hydroxylate ethylbenzene, which is a substrate for soluble but not particulate MMO (Burrows *et al.*, 1984). Similar findings have been reported by Jezequel & Higgins (1983) and Jezequel *et al.* (1984). These reports suggest that soluble MMO can indeed exist in methanol-grown *M. trichosporium* OB3b, although particulate MMO has not been reported.

The present study investigates the effect of growth conditions, in particular copper availability, on the intracellular location of MMO in *M. trichosporium* OB3b during growth on methanol. Our results show that this organism contains soluble or particulate MMO (or both) during growth on this carbon source.

METHODS

Organism and growth conditions. *Methylosinus trichosporium* OB3b was grown on a nitrate salts medium (NSM) (Cornish *et al.*, 1984). CuSO_4 was added to a final concentration of $1 \mu\text{M}$ unless otherwise stated. The components of the growth medium were dissolved in glass-distilled water. Water purified by reverse-osmosis followed by treatment with activated charcoal, resin ion exchange and ultrafiltration ($2 \mu\text{m}$) (Elga) was used as indicated.

M. trichosporium OB3b was first grown on NSM agar with methane as the carbon source, and adapted to grow on methanol (1%, v/v) at 30°C in shake-flask culture as described previously (Best & Higgins, 1981; Cornish *et al.*, 1984) except that the NSM medium was supplemented with filter sterilised NaHCO_3 (0.05%, w/v) as this was found to assist initial growth (all cultures from 12 trials grew on methanol in the presence of NaHCO_3 , whereas 80% failed to grow in its absence). Growing cultures were used to inoculate the fermenter.

The organism was grown on methanol (1%, v/v) in carbon-excess continuous culture using a 4 l capacity LKB Ultraform fermenter (temperature, 30°C ; impeller speed, 300 r.p.m.; $D = 0.04 \text{ h}^{-1}$). The growth limiting nutrient was varied as stated in the text.

Measurement of dissolved oxygen. This was done using an Orbisphere 2607 instrument fitted with a model 2111 sensor, connected to a chart recorder. The probe was sterilized by immersion in an aqueous solution of ethylene oxide (20%, v/v) overnight before insertion into the fermenter vessel. The instrument was calibrated in air-saturated water according to the manufacturer's instructions.

Preparation of whole-organism suspensions and cell-free extracts. These were prepared as described by Burrows *et al.* (1984).

Assay of MMO activity. All assays were carried out as described by Burrows *et al.* (1984). MMO activity of whole-organism suspensions was measured by following the epoxidation of propene (20 mM-sodium formate was used as a source of reducing equivalents) using gas chromatography. MMO activity in soluble and particulate fractions of cell-free extracts was also measured by following the epoxidation of propene but NADH (5 mM) was used as electron donor.

Measurement of dry weight and protein. Suspensions of *M. trichosporium* OB3b were washed once using distilled water and dried at 100 °C to constant weight. Protein content of cell-free preparations was measured using the modified Lowry assay of Peterson (1977). Bovine serum albumin was used as standard.

SDS-PAGE. Electrophoresis was carried out on 12% (w/v) acrylamide gels using the discontinuous buffer system of Laemmli (1970). Proteins were fixed, stained with Coomassie blue and the gels destained as described by Laemmli (1970). The following proteins were used as standards (M_r in parentheses): lactalbumin (14 200); trypsin inhibitor (20 100); trypsinogen (24 000); carbonic anhydrase (29 000); glyceraldehyde-3-phosphate dehydrogenase (36 000); egg albumin (45 000); bovine albumin (66 000). Methanol dehydrogenase from *M. trichosporium* OB3b (M_r 60 000) was used as a further standard. This was a gift from Dr D. J. Best, Cranfield Institute of Technology.

Chemicals. All chemicals were obtained from BDH except for M_r standards, which were purchased from Sigma.

RESULTS

Effect of growth conditions on the intracellular location of MMO

M. trichosporium OB3b was initially grown on methanol (1%, v/v) in continuous culture ($D = 0.04 \text{ h}^{-1}$; air flow rate, 100 ml min^{-1}) on NSM medium prepared using purified water (see Methods). The normal concentration of Cu^{2+} was $1 \mu\text{M}$. Under these growth conditions the culture was O_2 -limited and 34–57% of the total MMO activity was located in the soluble fraction of cell-free extracts (Table 1). The proportion of soluble MMO could be increased by imposing a copper limitation on the organism: this was accomplished either by omitting CuSO_4 from the growth medium, in which case the biomass density of the culture decreased, or by increasing the aeration rate (and hence O_2 availability), in which case the biomass density rose by a small but significant amount prior to onset of copper limitation (Table 1). For a given aeration rate, the proportion of particulate MMO increased only when the copper concentration was raised and the culture allowed to become copper-sufficient (Table 1). The same pattern of MMO regulation was observed when *M. trichosporium* OB3b was grown on NSM medium prepared using glass-distilled water, except that the biomass densities of steady state cultures were significantly lower for a given amount of added copper (Table 1). This was presumably because the copper content of the glass-distilled water was lower than that of the purified water used in previous experiments.

Table 1. *Effect of growth conditions on the intracellular location of MMO in methanol-grown M. trichosporium* OB3b

M. trichosporium OB3b was grown on methanol (1%, v/v) in continuous culture ($D = 0.04 \text{ h}^{-1}$). The amount of CuSO_4 added to the medium feed and the availability of O_2 were varied as indicated. The growth medium was prepared using either purified water (a) or glass-distilled water (b) (see Methods). Most data are given as the mean $\pm$ SEM of measurements carried out on separate days; the numbers of independent determinations are given in parentheses.

	CuSO_4 (μM)	Growth- limiting nutrient	Air flow rate (ml min^{-1})	Biomass density (g l^{-1})	Distribution of cell-free MMO activity (% of total)*		Whole-cell MMO activity [nmol propene oxide formed min^{-1} (mg dry wt) $^{-1}$]
					Soluble fraction	Particulate fraction	
(a)	0	Copper	100	0.93 ± 0.05 (5)	85–92	8–15 (3)	12.7 ± 4.7 (5)
	1	Oxygen	100	1.2 ± 0.03 (7)	34–57	43–66 (7)	32.5 ± 13.6 (5)
	1	Copper	400	1.35 ± 0.03 (3)	85	15 (1)	17.6 (1)
	4	Oxygen	100	1.2 ± 0.03 (5)	11	89 (1)	18.9 (1)
	4	Copper	400	1.8 ± 0.06 (3)	39	61 (1)	28.8 (1)
(b)	0	Copper	100	0.64 ± 0.03 (4)	83–100	0–17 (3)	NT
	0	Copper	400	0.61 ± 0.01 (3)	95	5 (1)	NT
	1	Oxygen	100	0.70 (2)	0	100 (1)	NT
	1	Oxygen	400	0.89 ± 0.05 (3)	48	52 (1)	NT

NT, Not tested.

* 100% MMO activity is defined as the activity found in the soluble fraction of cell-free extracts, measured by following propene oxidation, plus that found in the particulate fraction of the same sample.

Fig. 2. Oxidation of propene and ethylbenzene by *M. trichosporium* OB3b following relief of copper deficiency. The organism was initially grown on methanol (1%, v/v) in continuous culture under copper limitation ($D = 0.04 \text{ h}^{-1}$; air flow rate, 100 ml min^{-1} ; biomass density, 0.9 g l^{-1} ; NSM medium, nominally copper-free, prepared using purified water). Under these conditions 92% of the MMO activity was located in the soluble fraction of cell-free extracts. Particulate MMO was induced by adding $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ to the culture vessel to give a final concentration of $4 \mu\text{M-Cu}^{2+}$; the Cu^{2+} concentration of the medium feed was increased to $4 \mu\text{M}$ at the same time. Samples were withdrawn from the chemostat at intervals following copper addition and rates of oxidation of propene and ethylbenzene by whole organisms were measured by following product formation using gas chromatography. ○, Propene oxide; ●, 1-phenylethanol; □, 4-hydroxyethylbenzene.

As expected, the ambient concentration of O_2 in copper-limited cultures rose as the aeration rate was increased, whereas the O_2 concentration was below the limit of detection in copper-sufficient, O_2 -limited cultures (Table 1). Interestingly, the dissolved O_2 concentration of a copper-limited culture ($D = 0.04 \text{ h}^{-1}$; biomass density, 0.61 g l^{-1} ; no added Cu^{2+} ; air flow rate, 100 ml min^{-1} ; NSM medium prepared using glass-distilled water) decreased exponentially with time ($t_{1/2} = 45 \text{ min}$) from 5.4 to $1.1 \mu\text{M}$ within 2 h of adding CuSO_4 ($1 \mu\text{M}$). The kinetics of this decay suggest that during copper-limited growth the organism may contain a protein that is potentially capable of reducing molecular O_2 and which is activated when additional copper is made available (see also Prior & Dalton, 1985).

MMO activity during growth on methanol

Whole cells of *M. trichosporium* OB3b retained MMO activity during growth on methanol under all conditions tested, as judged from their capacity to oxidize propene to propene oxide in the presence of sodium formate (Table 1). The rates of propene oxide formation were, however, significantly lower than those reported for washed whole-cell suspensions of methane-grown *M. trichosporium* OB3b [i.e. $90\text{--}160 \text{ nmol propene oxide formed min}^{-1} (\text{mg dry weight})^{-1}$] (Burrows *et al.*, 1984; Cornish *et al.*, 1984). Organisms that had been grown under conditions of copper deficiency and contained soluble MMO were, in addition, capable of oxidizing ethylbenzene (which is a substrate for the soluble but not particulate MMO) to 4-hydroxyethylbenzene and 1-phenylethanol, but this capacity was progressively lost when additional copper was introduced to the growth medium and MMO activity transferred to the particulate fraction of cell-free extracts (Fig. 2). The rate of propene oxidation by whole-cell suspensions altered little during this time (Fig. 2). The change in intracellular location of MMO was accompanied by the disappearance of four abundant, soluble polypeptides (M_r 57 000, 45 000, 23 000 and 16 500) and the concomitant increase in the concentration of a membrane-bound polypeptide (M_r 42 000) (Fig. 3). The concentration of these polypeptides changed in essentially the same fashion following the addition of copper to cultures of *M. trichosporium* OB3b growing on methane under

Fig. 3. Changes in the concentrations of cellular polypeptides following addition of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ to a copper deficient culture of *M. trichosporium* OB3b. The figure, a photograph of an SDS-PAGE gel, shows the protein profiles of whole cells of *M. trichosporium* OB3b withdrawn from the chemostat during the experiment described in the legend to Fig. 2. Numbers above the gel tracks denote times of sampling (h) following addition of copper. Track B contained M_r standards, track A pure methanol dehydrogenase of *M. trichosporium* OB3b.

conditions of copper deficiency, except that the soluble protein of M_r 16500 was not noted previously (Burrows *et al.*, 1984). The precise function of these polypeptides is at present uncertain. Three of the soluble polypeptides (M_r 57000, 45000, and 23000), however, are almost identical in size to the three hydroxylase subunits of soluble MMO isolated from *Methylococcus capsulatus* (Bath) (Woodland & Dalton, 1984) and *Methylobacterium* sp. strain CRL-26 (Patel, 1984) and may well be constituents of the corresponding enzyme from *M. trichosporium* OB3b.

M. trichosporium OB3b was grown on methanol in continuous culture for up to 1600 h (approximately 90 generations). The organism retained the capacity to grow on methane without discernible lag after this time, indicating that the methane-oxidizing system(s) were still functional despite prolonged cultivation on an alternative carbon source.

DISCUSSION

The objective of the present work was to establish whether *M. trichosporium* OB3b retains the capacity to express two types of MMO (i.e. soluble and particulate) during growth on methanol. Our results show that this is indeed the case and indicate that copper availability governs which type of MMO predominates during growth of *M. trichosporium* OB3b on methanol as well as on methane (Stanley *et al.*, 1983; Burrows *et al.*, 1984). *M. trichosporium* OB3b is the only methanotroph in which both types of MMO have been found to occur during growth on methanol. Intracellular location of MMO may be regulated differently in *Methylococcus capsulatus* (Bath) during growth on methanol, however, since this organism expressed only particulate MMO during growth on methanol, even when copper was omitted from the growth medium (Prior & Dalton, 1985).

The precise mechanism(s) by which copper availability regulates MMO remains to be established, but it is worth noting that one of the components of the particulate MMO of *M. trichosporium* OB3b was reported to be a membrane-bound copper protein (*M*, 46000 estimated using SDS-polyacrylamide disc gel electrophoresis) (Tonge *et al.*, 1977). This MMO was reported to function in cell-free preparations with ascorbate as electron donor (Tonge *et al.*, 1975) but this finding has not been substantiated (Scott *et al.*, 1981). Furthermore, subsequent attempts to solubilize the particulate MMO of *M. trichosporium* OB3b (Burrows *et al.*, 1984) and other methanotrophs have resulted in complete loss of activity (Colby *et al.*, 1975; Dalton & Leak, 1985) and it has not proved possible, to date, to purify the enzymes in an active form for further characterization. Prior & Dalton (1985) observed that particulate MMO activity was stimulated when Cu^{2+} ions were added to cell-free extracts of *Methylococcus capsulatus* (Bath), which suggests that this MMO and/or one of the associated electron transfer proteins probably contains copper. The involvement of a copper protein would, of course, account for the finding that the particulate MMOs of *M. trichosporium* OB3b and *Methylococcus capsulatus* (Bath) are active only when the organisms are grown under conditions of copper sufficiency.

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