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Recent Progress in Research on Methanotrophs and Methane Monooxygenases

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Introduction

Micro-organisms capable of growth on C_1 compounds as sole carbon and energy sources are classified as methylotrophs (*see* Tani, 1985), while those of them that are capable of growth on methane gas are called methanotrophs. They contrast with methanogenic bacteria which produce methane from organic compounds. The obligate methanotrophs have been classified by Whittenbury, Phillips and Wilkinson (1970) into two types according to their pathway of carbon assimilation and internal membrane structure: type I methanotrophs have a ribulose monophosphate (RMP) pathway, a GC content of 50–54%, and bundles of vesicular discs of membrane distributed throughout the organism; type II methanotrophs have a serine pathway, a GC content of 62·5%, and paired membranes around the periphery of the cell. Further studies on the type I organism *Methylococcus capsulatus* Bath have revealed the presence of some type II and some autotrophic characteristics; this strain therefore has been assigned to a third group, type X (Whittenbury and Dalton, 1981). Another class of micro-organisms, facultative methanotrophs that utilize not only methane but also multicarbon compounds, was first described by Hanson and his colleagues (Patt *et al.*, 1974).

Prior to 1982, several reviews on methylotrophs (including methanotrophs) appeared (Quayle, 1972; Colby, Dalton and Whittenbury, 1979; Wolfe and Higgins, 1979; Dalton, 1980; Hanson, 1980; Higgins, 1980; Hou, Patel and Laskin, 1980; Higgins *et al.*, 1981; Hou, 1982). In 1982, an excellent book was published by Anthony on the biochemistry of methylotrophs, which is regarded as the definitive textbook on this subject. Another book covering all aspects of methylotrophs, written by both academic and industrial scien-

Abbreviation: MMO, methane monooxygenase

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tists, gives a thorough analysis of both basic and applied aspects (Hou, 1984a). However, many papers have appeared since these books and articles were published.

The purpose of this chapter is to review in detail the recent information on methane-utilizing bacteria. The oxidative pathway of methane in methanotrophic bacteria involves a series of two electron oxidation steps including the enzymes methane monooxygenase (EC 1.14.13.25), methanol dehydrogenase (alcohol oxidase; EC 1.1.3.13), formaldehyde dehydrogenase (EC 1.2.1.46), and formate dehydrogenase (EC 1.2.1.2) (*Figure 1*). The enzymes catalysing each step have been detected in cell-free systems, and have been purified from many strains. This chapter reviews only those reports involving the first enzyme, i.e. methane monooxygenase (MMO), which catalyses the initial attack on the methane molecule. Areas of discrepancies among reports are stressed. Methylotrophs that utilize C_1 compounds but not methane gas are excluded from this review.

Location of methane monooxygenase

Early reports on methane monooxygenase described the enzyme activity associated with membrane fractions of methanotrophs. Colby and Dalton (1976) first reported a soluble methane-oxidizing system from *Methylococcus capsulatus* (Bath). Since then, the location of MMO inside the cells has become a controversial issue which has not yet been fully resolved. To give the reader a better understanding of this issue, papers dealing with the cellular location of MMO activity are here reviewed chronologically.

Leadbetter and Foster (1959) were the first to propose a monooxygenase mechanism for methane oxidation:

$$CH_4 + O_2 + XH_2 \rightarrow CH_3OH + H_2O + X$$

They demonstrated that *Pseudomonas methanica* grown in the presence of an atmosphere enriched in ^{18}O incorporated 16 times as much ^{18}O into cell material when methane, rather than methanol, was the carbon source. Higgins and Quayle (1970) provided direct evidence for oxygenase involvement in the oxidation of methane. They isolated $CH_3\ ^{18}OH$ as the product of methane oxidation when suspensions of *P. methanica* or *Methanomonas methanoxidans* were allowed to oxidize methane in an $^{18}O_2$-enriched atmos-

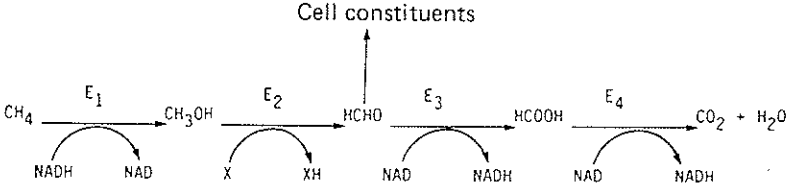


Figure 1. Methane oxidative pathway. E_1: methane monooxygenase; E_2: methanol dehydrogenase or alcohol oxidase; E_3: formaldehyde dehydrogenase; E_4: formate dehydrogenase.

phere. Studies of MMO at the cell-free level were achieved first by Ribbons and Michalover (1970), who demonstrated methane oxidation by cell-free extracts from *Methylococcus capsulatus*. They observed methane-stimulated respiration and NADH oxidation with stoichiometry consistent with a monooxygenase mechanism. The methane-stimulated oxidation of NADH resided mostly in the large particle fractions. This fraction contained most of the intracytoplasmic membranes that are so characteristic of *Methylococcus capsulatus*; it was free of whole cells, but contained some cell-wall materials. Ribbons and Michalover did not consider that the finding of particulate MMO was surprising, as bacteria that are able to oxidize methane are peculiar in possessing extensive intracytoplasmic membrane systems. Later, in more detailed studies, Ribbons (1975) reported that the particulate fractions that catalysed the methane-stimulated oxygen consumption and NADH oxidation also catalysed the oxidation of methanol and formaldehyde with oxygen as the terminal electron acceptor. Formate was detected as the product of methane oxidation. Ribbons also found considerable variability in the MMO activities of individual preparations, with many showing very little activity. Methane-dependent NADH oxidation was inhibited by electron transport inhibitors such as azide, cyanide, British anti-Lewisite, antimycin, and *o*-phenanthroline.

Another particle-bound MMO was reported by Ferenci (1974) and Ferenci, Strom and Quayle (1975): from *Pseudomonas methanica* they isolated cell-free particulate fractions that were capable of catalysing NADH-dependent oxygen consumption using methane and carbon monoxide as substrates. Stoichiometric conversion of carbon monoxide to carbon dioxide involving a monooxygenase reaction was demonstrated and evidence was presented indicating that methane and carbon monoxide oxidation are catalysed by a single enzyme system. These particulate fractions also catalysed the oxidation of ethane. Oxidation of methane and carbon monoxide was competitively inhibited by ethane and ammonium chloride. The activity of this system was very unstable, with complete loss of activity occurring after storage at 0°C for 24 hours. The activity was inhibited by dithiothreitol, reduced glutathione and cyanide.

Colby, Dalton and Whittenbury (1975) reported another particulate-bound MMO activity from *Methylomonas methanica*: this enzyme catalyses the O_2- and NAD(P)H-dependent disappearance of bromomethane and the oxidation of methane. The activity of this particulate bromomethane monooxygenase is inhibited by metal-binding reagents, by electron transport inhibitors, by some metal ions and by acetylene.

Particulate MMO activity was also reported by Tonge *et al.* (1974, 1975). They detected methanol accumulation in the presence of 150 mM phosphate buffer in cell-free extracts of *Methylosinus trichosporium* OB3b. All the methane-oxidizing activity was located in particulate fractions that sedimented at 150 000 *g* for 90 minutes. Methanol, NADH and ascorbate were equally effective electron donors to the enzyme. The enzyme activity was not stable on freezing and was sensitive to cyanide, a variety of metal-chelating agents and electron transport inhibitors. The data of Tonge *et al.*

suggested that NADH was not the immediate electron donor, but that electrons are passed from NADH along an electron-transport chain to the physiological donor. This enzyme system appears to resemble that reported in the *Methylococcus capsulatus* Texas strain (Ribbons, 1975). All the papers published prior to 1975 on MMO indicate that the enzyme system is particulate in nature. Our studies (Hou *et al.*, 1979; Patel *et al.*, 1979) also showed that MMO activity was associated with the particulate fraction of methanotrophs when cells were grown in shake flasks.

In contrast to the particle-bound MMO systems described above, Colby and Dalton (1976) found that the MMO system from *Methylococcus capsulatus* Bath resides exclusively in the soluble fractions of the cell-free extract (i.e. it is not sedimented at 160000*g* for 1 hour). This soluble extract catalyses the NAD(P)H- and oxygen-dependent disappearance of bromomethane, and also the formation of methanol from methane. This behaviour differs from that of the particulate MMO system reported by the same investigators in *Methylomonas methanica* (Colby, Dalton and Whittenbury, 1975).

Subsequently, Stirling and Dalton (1979) reported a soluble MMO system from *Methylosinus trichosporium* OB3b, with properties similar to those described for *Methylococcus capsulatus* Bath. Scott, Best and Higgins (1981) and Scott, Brannon and Higgins (1981) confirmed that a soluble MMO system was produced when strain OB3b was grown under methane- or nitrate-limiting conditions. Under oxygen-limiting conditions, the organism possessed extensive intracytoplasmic membranes and the MMO activity was particulate, whereas under methane- or nitrate-limiting conditions few intracytoplasmic membranes were seen and the MMO system was soluble. Patel *et al.* (1982) also reported a soluble MMO system from a facultative *Methylobacterium* sp. CRL 26 when this was grown in a fermenter where the oxygen was not limited, instead of in shake flasks. However, the soluble MMO system obtained by Stirling and Dalton (1979) from strain OB3b was from oxygen-limited chemostat cultures. There may therefore be other factor(s) controlling the cellular location of the MMO system in methanotrophs.

Recently, Dalton and his co-workers (Stanley *et al.*, 1983; Dalton *et al.*, 1984) added another aspect to the controversy concerning the intracellular location of the MMO system. They reported that the intracellular location of methane monooxygenase activity in the methanotroph *Methylococcus capsulatus* Bath depends primarily on the availability of copper. MMO activity was observed in the particulate fraction of cell extracts under conditions of copper excess (1·2 mg copper sulphate/litre), but switched to a soluble location in response to copper stress (less than 0·2 mg copper sulphate/litre). The cell density of methanotrophs also is a possible determinant of the cellular location of the MMO system: at a low cell concentration (about 0·8 g (dry weight) per litre) MMO was entirely particulate but, as cell density increased, MMO could be detected as a soluble enzyme. The ratio of soluble to particulate activity increased with increasing biomass until, at about 1·6 g cells (dry weight) per litre, MMO was found only in the soluble form. The two types of MMO activities could be differentiated by

sensitivity to a range of inhibitors, and by major changes in the polypeptide patterns on denaturing polyacrylamide gels. Examination of other methanotrophs showed that copper concentration also regulates the intracellular location of MMO in *Methylosinus trichosporium* OB3b but not in *Methylomonas albus* BG8 or *Methylocystis parvus* OBBP. The regulation of the MMO cellular location by copper content was confirmed by Burrows *et al.* (1984) and by Cornish *et al.* (1985). When *Methylosinus trichosporium* OB3b was grown in a continuous culture under conditions of copper deficiency (biomass density 1·6 g/litre; 1 μM Cu^{2+}), the cell exhibited a vesicular ultrastructure and contained only soluble MMO (Burrows *et al.*, 1984). When the copper content of the medium was raised to 10 μM, the number of intracytoplasmic membranes increased and the MMO was particulate. Cornish *et al.* (1985) also reported that the relief of copper deficiency during growth promotes the proliferation of intracytoplasmic membranes together with the synthesis of particulate MMO in *Methylosinus trichosporium* OB3b.

Thus, it seems that, during their growth, methanotrophs may possess two forms of MMO, regulated by the copper content of the growth environment. However, this raises several questions. First, are components A, B, and C (*see* 'Purified MMO systems', pages 153–160) of the soluble MMO, part of the membrane-bound MMO or are they different proteins from *de novo* synthesis triggered by copper stress? Second, what is the true role of copper? Is it one of the components of MMO, or does it play a part in determining the subcellular location of MMO? There is, as yet, no clear-cut answer to any of the above questions. To prove any relationship between protein components of the soluble and the particulate MMO systems, the components must be characterized. The great differences in the protein molecular weight, metal content and electron transfer system reported between the particulate (Tonge, Harrison and Higgins, 1977) and soluble (Dalton, 1980) MMO systems indicate that it is highly probable that they are completely different.

Hubley, Thomson and Wilkinson (1975) first noticed a possible role for copper in methane monooxygenase on the basis of the pattern of inhibition and its relief by added metal ions. Ohtomo, Iizzuka and Takeda (1977) and Takeda and Tanaka (1980) showed that, in methane-grown *Methanomonas margaritae* in the presence of copper (1 mg/litre), cells possessed intracytoplasmic paired membranes along the periphery of the cell whereas, in the absence of copper, the cells possessed large vesicles. Similar phenomena were observed by Burrows *et al.* (1984) and Cornish *et al.* (1985) for *Methylosinus trichosporium* OB3b. Dalton *et al.* (1984) speculated that this dramatic morphological difference induced by copper may have a role in determining the subcellular location of MMO. However, there is no undisputed relationship between MMO and the intracytoplasmic membranes which are induced by copper during cell growth. In addition, copper has been reported to be one of the components of particulate MMO (Tonge, Harrison and Higgins, 1977). The true role of copper in MMO systems requires further investigation, the results of which may help to explain the biochemical and metabolic significance of the two forms of MMO in methanotrophs.

In organisms other than methanotrophs, soluble octane monooxygenase (alkane 1-monooxygenase; EC 1.14.15.3) has been purified from octane-grown *Pseudomonas oleovorans* (McKenna and Coon, 1970). On the other hand, a particulate octane monooxygenase system has been partially purified from *Corynebacterium* sp. strain 7E1C (Cardini and Jurtshuk, 1970). Interestingly, these micro-organisms do not possess large amounts of intracytoplasmic membranes: the existence of both soluble and particulate MMO systems among methanotrophs may not, therefore, be so surprising.

Regulation of MMO synthesis by methanol

Methanol is extremely toxic to most methanotrophs (with the exception of the *Methylomonas* group) when added to the medium at concentrations as low as 0·01% (v/v) (Whittenbury, Phillips and Wilkinson, 970). A method for the adaptation of the methanotroph *Methylocystis parvus* OBBP to methanol at concentrations of up to 4% (w/v) has been described (Hou *et al.*, 1978). Linton and Vokes (1978) reported that the ability of *Methylococcus* NCIB 11083—a type I methanotroph—to use methane, was lost after successive transfers in shake flask cultures with methanol at a concentration of 0·1% (v/v). We found that many methanotrophs gradually lost their methane-utilizing ability after being adapted to grow on methanol through successive transfers in shake flasks. These cultures failed to grow on methane and possessed negligible alkane hydroxylation and alkene epoxidation activities. These findings suggest that the appropriate enzyme system (MMO) may be inducible or plasmid bound (Patel, Hou and Felix, 1978; Hou *et al.*, 1979). Lynch, Wopat and O'Connor (1980) also found that *Methylobacterium hypolimneticum*, a facultative methanotroph, had no methane-oxidizing ability when the cells were grown on heterotrophic substrates; only methane-grown cells contained intracytoplasmic membranes. However, Takeda *et al.* (1976) reported that *Methanomonas margaritae* cells grown on methanol in batch culture showed methane-stimulated oxygen uptake rates that were about half the rates observed when cells were grown on methane. Polarographic studies (Best and Higgins, 1981), on washed cell suspensions of *Methylosinus trichosporium* OB3b derived from batch cultures grown on methanol at high concentrations, revealed that the cells required copious washing to remove the residual methanol, which was largely responsible for apparently high endogenous respiration rates. Methanol has been reported to be a substrate (Colby, Stirling and Dalton, 1977) and a competitive inhibitor of MMO (Colby, Dalton and Whittenbury, 1975): the inability of methanol-grown methanotroph cells to hydroxylate methane or to epoxidize alkenes, observed by some research groups (Patel, Hou and Felix, 1978; Hou *et al.*, 1979; Lynch, Wopat and O'Connor, 1980), may therefore be due to the presence of residual methanol. To overcome this possibility, Best and Higgins (1981) preincubated methanol-grown cells in an open-topped oxygen electrode for 30 minutes to allow residual methanol to be metabolized. In this way, they detected methane-stimulated oxygen uptake by methanol-grown cells that was approximately 40% of that measured for methane-grown cells. A similar

rate of MMO activity was observed for methanol-grown cells harvested from steady-state continuous culture. MMO from methanol-grown cells was located in the soluble fraction and could be linked only to NAD(P)H. Its specific activity was similar to that of the soluble enzyme in extracts of methane-grown organisms (20–30 nmol/min/mg protein). These findings still cannot rule out the substrate induction hypothesis for MMO systems, because both methane and methanol are good substrates for MMO (Colby, Stirling and Dalton, 1977). Recently, Dalton *et al.* (1984) found that when chemostat cultures of *Methylococcus capsulatus* Bath were switched from growth on methane to growth on formaldehyde, there was a loss of alpha, beta, and gamma subunits (*see* 'Purified MMO systems', page 156) of protein A as judged by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Accompanying this loss of soluble MMO protein A was a decrease in the methane-stimulated oxygen uptake rate. As there was no switch of MMO from soluble to particulate under these experimental conditions, the authors suggest that synthesis of the soluble MMO is induced by methane or methanol.

Despite all of these data, many questions still remain unanswered. Does the low level of MMO or methane-stimulated oxygen uptake rate in methanol-grown cells (40% of that for methane-grown cells; Best and Higgins, 1981), or in formaldehyde-grown cells (25% of that for methane-grown cells; Dalton *et al.*, 1984) represent a basal constitutive level of MMO present under all growth conditions? If MMO is constitutive, then how does one explain the loss of ability to use methane in those cells cultured in successive transfers on a medium with a high level of methanol? Is a plasmid involved in methane oxidation? As methanol-grown cells had few, if any, intracytoplasmic membranes, does this mean that MMO in methanol-grown cells must be the soluble form? Is there a general answer to the above questions or does each strain behave differently? Further studies are needed to resolve all of these mysteries.

Electron donor for MMO

The first report on an electron donor for MMO was published by Ribbons and Michalover (1970), who observed the methane-stimulated NADH oxidation catalysed by cell-free extracts of *Methylococcus capsulatus*. Later, in more detail, Ribbons (1975) described the methane-stimulated oxygen consumption and NADH oxidation by particulate fractions derived from *Methylococcus capsulatus* Texas. The activity resided mainly in large particles which contained intracytoplasmic membranes. Formate was detected as the product of methane oxidation. The particulate fractions also catalysed the oxidation of methanol and formaldehyde. Ferenci (1974) and Ferenci, Strom and Quayle (1975) also isolated cell-free particulate fractions from *Methylomonas methanica* that were capable of catalysing NADH-dependent oxygen consumption using methane and carbon monoxide as a substrate; these particulate fractions also catalysed the oxidation of ethane. The MMO activity in whole cells was stimulated by ethanol, despite the absence of an

NAD^+-linked alcohol dehydrogenase in methanotrophs (*Figure 1*). They suggested that NADH may be generated indirectly from ethanol oxidation by reversed electron flow. By studying ethane oxidation by methanotrophs, Hazeu and de Bruyn (1980) suggested that alcohol dehydrogenase could supply reductant for the MMO system *in vivo* in some methanotrophs. The *in vitro* use of ascorbate and methanol as electron donors for MMO (Tonge, Harrison and Higgins, 1977) from *Methylosinus trichosporium* OB3b could not be substantiated (Stirling and Dalton, 1979; Scott, Best and Higgins, 1981; Scott, Brannon and Higgins, 1981; Patel *et al.*, 1982). The soluble MMO purified from *Methylococcus capsulatus* Bath utilized NAD(P)H (Colby and Dalton, 1978; Woodland and Dalton, 1984; Lund, Woodland and Dalton, 1985). Compounds which, when oxidized, yield NADH directly in whole cells (Hou *et al.*, 1980; Leak and Dalton, 1983) or in cell-free systems with a corresponding dehydrogenase (Hou *et al.*, 1982) can act as electron donors for the MMO system. Methanol was successfully demonstrated to regenerate cofactor used by MMO in cells packed in a heterogeneous bioreactor (Hou, 1984b).

The only authentic electron donor for soluble MMO *in vitro* appears to be NAD(P)H, but the electron donor for MMO *in vivo* or in cell-free particulate MMO fractions has not yet been positively identified.

In addition to the above-mentioned reports describing stimulation of MMO activity *in vivo* by compounds other than NADH (Ferenci, Strom and Quayle, 1975; Tonge *et al.*, 1977; Hazeu and de Bruyn, 1980), there have recently been several papers dealing with the electron donor for MMO in whole cells of methanotrophs. Stanley *et al.* (1983) found that ethanol-driven MMO activity was observed only in cells with particulate MMO activity. Leak and Dalton (1983) conducted *in vivo* studies of primary alcohols, aldehydes and carboxylic acids as electron donors for MMO in a variety of methanotrophs: they found that C2 to C4 primary alcohols and their corresponding aldehydes were oxidized by obligate methanotrophs; reducing equivalents from each oxidation step could be utilized, *in vivo*, to stimulate MMO activity. As none of these oxidation steps produces NADH directly, the authors present these observations as evidence for reverse electron transport in methanotrophs.

Most recently, Cornish *et al.* (1985) found that succinate acted as electron donor *in vitro* for the particulate MMO of *Methylosinus trichosporium* OB3b. Succinate did not support propylene epoxidation when cell-free extracts of strain OB3b contained only soluble MMO. Succinate dehydrogenase is also membrane-bound. Various experimental findings led these authors to conclude that succinate-linked MMO activity is NADH independent. This supports previous hypotheses that particulate MMOs can obtain electrons *in vivo* from the respiratory chain and NAD-independent dehydrogenases such as methanol dehydrogenase (Tonge *et al.*, 1975; Leak and Dalton, 1983).

Purified MMO systems

MMO SYSTEM FROM *METHYLOSINUS TRICHOSPORIUM* OB3b

Fractionation of cell-free extracts has revealed that methane monooxygenase consists of three components (Tonge *et al.*, 1977): one is soluble and the other two are particulate but can be solubilized by treatment with phospholipase D. The soluble CO-binding cytochrome *c* (a redox potential of +310 mV) has an oxidase function (Tonge *et al.*, 1975). The two proteins solubilized from the particulate fraction are a copper-containing protein and a small protein. Their physical properties are listed in *Table 1* together with those from other MMO systems. The pure MMO has a high specific activity (6 μmol/min/mg protein) and is fairly stable in storage at 0–40°C, but is inactivated upon freezing. The system is absolutely dependent on cytochrome c_{CO} from *Methylosinus trichosporium*. Because the monooxygenase is a copper-containing enzyme, the observed inhibition by metal-chelating agents is not surprising. The system is also inhibited by 2-mercaptoethanol and dithiothreitol. The purified enzyme system utilizes ascorbate or methanol (in the presence of methanol dehydrogenase) as electron donors. The enzyme cannot be linked directly to NADH, however. Tonge *et al.* (1977) have suggested that electrons may have been derived from NADH via an electron transport chain to the physiological donor. They proposed that, in *Methylosinus trichosporium* OB3b, ascorbate was probably directly reducing a carbon monoxide-binding cytochrome *c* which was essential for monooxygenase activity. The purified monooxygenase system from *Methylosinus trichosporium* OB3b catalysed the oxidation of ethane, propane, butane and carbon monoxide, in addition to that of methane. The biochemical properties of this enzyme system are listed in *Table 2*.

MMO SYSTEM FROM *METHYLOCOCCUS CAPSULATUS* BATH

The MMO system from *Methylococcus capsulatus* Bath (Colby, Stirling and Dalton, 1977) is located in the soluble fractions of the cell extract. NADH, but not ascorbate, acts as an electron donor. Unlike the particulate MMO system from strain OB3b, this MMO system was found to be relatively insensitive to a wide variety of inhibitors including cyanide, carbon monoxide, metal-chelating agents and electron transport inhibitors. This indicates that an electron transport chain was not involved in the transfer of electrons from NADH to monooxygenase. Acetylene and hydroxyquinoline strongly inhibited the monooxygenase activity. The soluble MMO system was resolved into three components and purified by ion exchange, affinity, and molecular sieve chromatographies (Colby and Dalton, 1978). All three components were unstable above 0°C; component A was reasonably stable at 0°C but components B and C were very unstable unless kept in the presence of phenylmethyl sulphonyl fluoride and sodium thioglycollate, respectively. However, all components were stable for at least three months if kept below

Table 1. Physical properties of MMO systems

Properties	*Methylosinus trichosporium* OB3b			*Methylococcus capsulatus* Bath			*Methylobacterium organophilum* CRL 26	
	Cytochrome C_{CO}	Protein 1	Protein 2	Compound C	Compound B	Compound A	Compound C	Compound A
Molecular weight	13 000	47 000	9 400	42 000	20 000	210 000	40 000	230 000
Subunit	—	—	—	—	—	54K 42K 17K $\alpha_2\beta_2\gamma_2$		55K 40K 20K $\alpha_2\beta_2\gamma_2$
Metal content	1 Fe 0·3–0·8 Cu	1 Cu	—	2 Fe 2S 1 FAD	None	2·3 Fe 0·2–0·5 Zn	2 Fe 2 S 1 FAD	2·8 Fe
Electron paramagnetic resonance	—	—	—	(reduced) g = 2·047 1·960 1·864 2·002	—	(reduced) g = 4·3, 2·01 1·934 (oxidized) g = 4·3 2.02	(reduced) g = 2·048 1·960	
S°_{20} (sedimentation coefficient)	9·8S					13·5S	2·1S	9·8S

Table 2. Biochemical properties of MMO systems

Property	*Methylosinus trichosporium* OB3b	*Methylococcus capsulatus* Bath	*Methylobacterium organophilum* CRL 26
Molar ratio of components for optimum activity	1:1:1	1:1:1	1:1
Specific activity	6 μmol/min/mg protein	0·072 μmol/min/mg protein	2·1 μmol/min/mg protein
pH optimum	6·9–7·0	—	7·0
PI	—	5·0–5·1 (compound A)	—
Electron donor	ascorbate	NADH, NADPH	NADH
Inhibitor	CN^-, metal binding and chelating agents, 2-mercaptoethanol, and dithiothreitol	8-Hydroxyquinoline Acetylene	Iodoacetamide and 5,5'-dithiobis-2-nitrobenzoate
Immunological properties	—	Compound A anti-sera does not cross-react with those from other methanotrophs	Antisera from flavoprotein and hydroxylase cross-react with corresponding proteins from other methanotrophs

−20°C. More data on this MMO system have been published recently. The three components of this MMO system are discussed in detail below.

Component A

Component A has previously been reported as having a molecular weight of about 220 000 daltons and subunits of molecular weight 68 000 and 47 000 (Dalton, 1980). The most purified form of the protein (95% as judged by gel electrophoresis) contains 2 gram-atoms (moles) of iron and acid-labile sulphide per mole of protein. Upon reduction with sodium dithionite (NADH is not effective), an electron paramagnetic resonance (EPR) signal appears which is enhanced fourfold in the presence of ethylene as substrate, suggesting that component A binds the substrate under reducing conditions. This oxygen-labile protein is purified under anaerobic conditions.

In a recent paper from Woodland and Dalton (1984), component A was further purified and characterized using a slightly modified purification method. Anaerobic chromatographic techniques did not enhance protein stability. Crude preparations of the enzyme appeared to be relatively stable (40% activity loss at 4°C over 72 hours). This loss of activity was not prevented by a variety of typical stabilizing agents, a methane or nitrogen atmosphere, or various oxygen-radical scavengers. None of the following conditions could enhance the stability of the enzyme: (1) the addition of 10% glycerol to the eluting buffer; (2) the use of a high pH (8·0) buffer; (3) the presence of a protease inhibitor. The inclusion of (0·1 mM) Fe^{2+}, Fe^{3+}, Zn^{2+}, Cu^{2+}, or Mo^{3+} in the enzyme assay did not stimulate the enzyme activity. 2-Mercaptoethanol was found to be strongly inhibitory. The purified protein can be stored at −20°C in 50% glycerol for several weeks without loss of enzyme activity.

As was stated by the authors, one important problem that remains to be overcome is the apparent loss of enzyme activity during purification. The specific enzyme activities of the crude extract and the purified form are the same, suggesting possibilities such as (1) no real protein purification; (2) the loss of iron, an essential cofactor, perhaps from the protein; (3) changes in protein structure; or (4) the involvement of another protein or cofactor. Experimental data showed that there was no involvement of another protein or cofactor, but the problem remains unsolved.

According to a recent report (Woodland and Dalton, 1984), component A has a molecular weight of 210 000 and comprises three subunits of 54 000, 42 000, and 17 000 which appear to be present in an $\alpha_2\beta_2\gamma_2$ arrangement. Component A is a virtually colourless protein, even at protein concentrations up to 50 mg/ml. In some preparations, a weak 'shoulder' of the absorption peak at around 410 nm was present; this 'shoulder' was lost upon reduction of the protein with sodium dithionite. The physical and biochemical properties of protein A are summarized in *Table 1* and *Table 2*, respectively.

Electron spin resonance (ESR) studies on component A suggest the possibility of a novel active centre in this enzyme (*Figure 2*). The oxidized protein gave two signals, one due to rhombic Fe^{3+} (g = 4·3) and an unusually broad

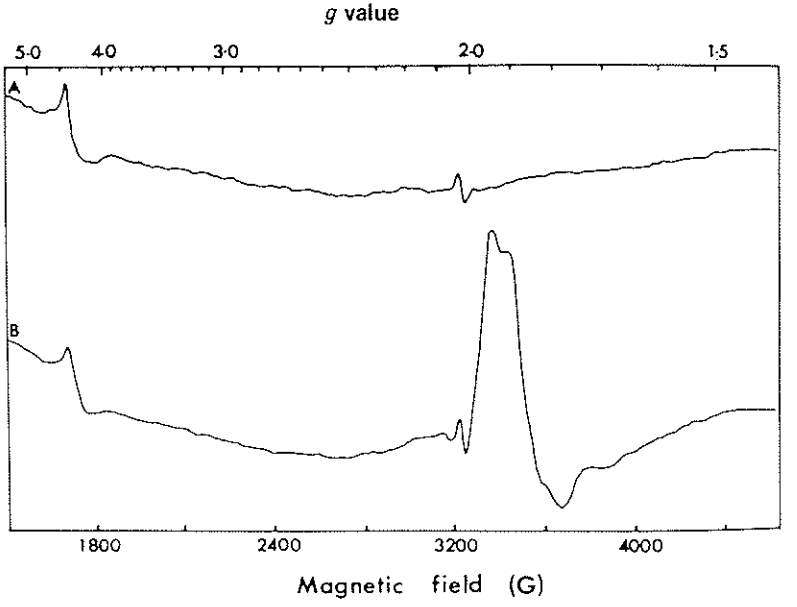


Figure 2. Electron spin resonance (ESR) spectrum of component A of MMO system from *Methylococcus capsulatus* Bath. Trace A, oxidized component A. Trace B, dithionite-reduced component A. (From Woodland and Dalton, 1984, with permission).

radical signal (g = 2·01). The reduced protein gave rise to an unusual signal below g = 2. The spectra do not resemble those obtained from typical Fe–S proteins (reduced Fe–S clusters have one g value above 2). An Fe–quinone complex was suggested.

Previously, it was reported that component A contains a single 2Fe–2S cluster (Dalton, 1980). However, a recent report (Woodland and Dalton, 1984) found no acid-labile sulphide in component A. Metal analyses of component A showed the presence of 2·3 moles of iron and 0·2–0·5 moles of zinc per mole of protein. There is no molybdenum, copper, nickel, or haem in the protein.

Component B

Component B is a colourless protein of molecular weight 15 000–20 000. It has no prosthetic groups and is not involved in intermolecular electron transfer (Lund, Woodland and Dalton, 1985); it has recently been purified (Green and Dalton, 1985). It is an acidic protein with an isoelectric point of 4·18 and has no absorption peak at visible wavelengths. Component B contains no acid-labile sulphide or metals such as iron, copper, zinc, manganese, magnesium or molybdenum, apart from a trace amount of nickel at 0·04 mole/mole. The sulphydryl group content is 1·16 mole/mole. Crude

component B is very stable at ambient temperatures in the presence of the protease inhibitor phenylmethylsulphonyl fluoride.

A role for component B in the MMO complex has been proposed (Green and Dalton, 1985): it is suggested to act as a regulatory protein of enzyme activity, possessing the capacity to convert the enzyme from an oxidase to an oxygenase (*Figure 3*). The reaction mechanism proposed is as follows: (1) components A and C catalyse the electron reduction of oxygen to water in the presence or absence of a hydroxylatable substrate, for example methane; (2) the addition of component B switches the enzyme complex from an oxidase to an oxygenase and the reduction of oxygen to water is no longer catalysed; (3) the addition of methane to the complete MMO complex restores interprotein electron transfer and the oxygenase reaction is catalysed.

Component C

Component C is an iron–sulphur flavoprotein of molecular weight 44 600, containing one FAD and one Fe_2S_2 centre per molecule (Colby and Dalton, 1978, 1979). In its oxidized form, component C is yellowish-brown with an absorption maximum at 465 nm. Component C was directly reduced by NADH under anaerobic conditions and was reoxidized anaerobically on the addition of a stoichiometric amount of component A. Component C is the

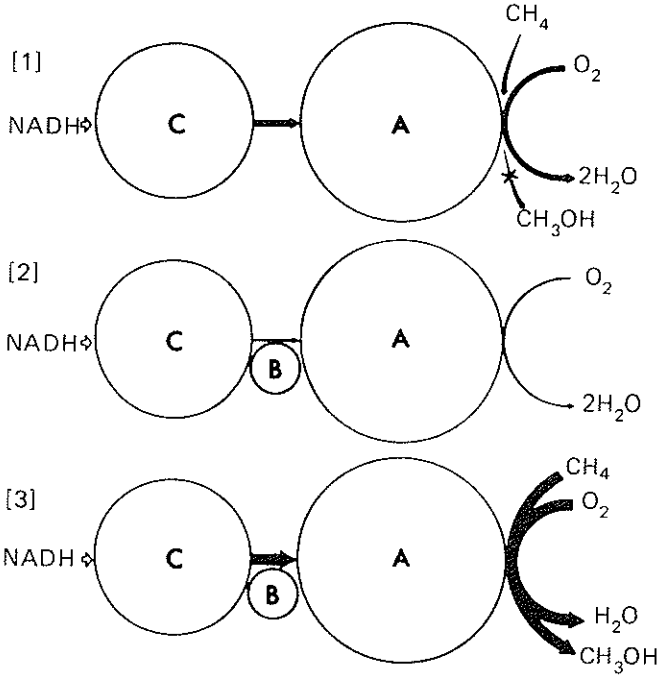
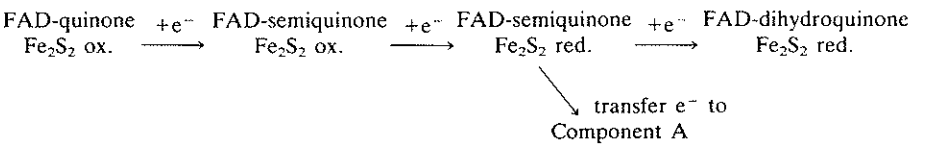


Figure 3. The role of component B in the soluble MMO complex from *Methylococcus capsulatus* Bath. (From Green and Dalton, 1985, with permission).

reductase component of the soluble MMO and is responsible for the transfer of reducing equivalents from NADH to component A.

The FAD and Fe_2S_2 redox centres of component C have been further characterized (Lund and Dalton, 1985; Lund, Woodland and Dalton, 1985). The Fe_2S_2 seems to be very similar to that of spinach ferredoxin, by its absorbance and EPR spectra, and the FAD semiquinone is a neutral semiquinone (*Figure 4*). Component C accepts electron pairs from NADH for passage to component A. The FAD appears to interact with NADH and transfers single electrons to the Fe_2S_2 for donation to component A at a constant redox potential. Data obtained from both EPR and spectrophotometry agree that the order of reduction of the redox centres in component C is as follows:



Removal and reconstitution of the redox centres of component C also suggests that the order of electron flow is:

$$\text{NADH} \rightarrow \text{FAD} \rightarrow \text{Fe}_2\text{S}_2 \rightarrow \text{component A}.$$

The reduction of component C by NADH is not rate-limiting for the methane monooxygenase reaction.

MMO SYSTEM FROM *METHYLOBACTERIUM* SP. CRL 26

The soluble MMO system from strain CRL 26 has been resolved into three protein fractions, A, B, and C, equivalent to those obtained from the Bath

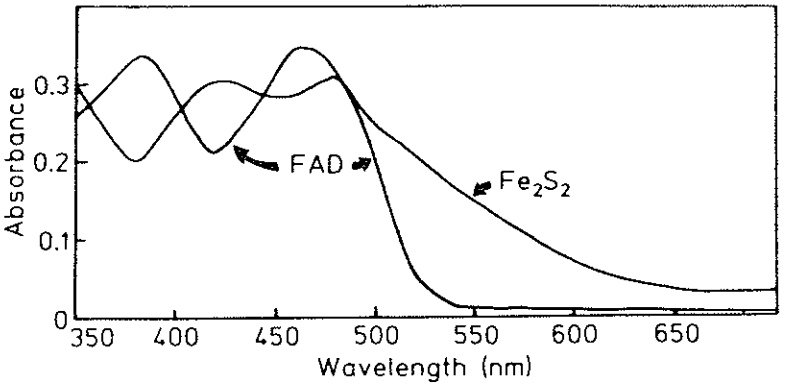


Figure 4. Resolution of the Fe_2S_2 and FAD redox centres of component C with mersalyl. (From Lund and Dalton, 1985, with permission).

strain (Patel *et al.*, 1982). Inhibition patterns differed for particulate (Patel *et al.*, 1979) and soluble MMO systems from strain CRL 26: cyanide and metal-binding compounds strongly inhibited the particulate MMO activity, whereas soluble MMO activity was not inhibited or only slightly inhibited by these compounds. The only potent inhibitors for soluble MMO activity were iodoacetamide and 5,5′-dithiobis-2-nitrobenzoate.

More recently, components of the soluble MMO system from strain CRL 26 have been purified (Patel, 1984). Only two components, analogous to the components C and A above, are required for MMO activity. The physical and biochemical properties of these two components are given in *Tables 1* and *2*.

Component A

Component A (hydroxylase) of the MMO system from strain CRL 26 was purified 27-fold to a constant specific activity of 2·1 μmol/min/mg protein (Patel, 1984). It has a molecular weight of 230 000 and contains three non-identical subunits of molecular weight 60 000, 40 000 and 20 000. Component A contains 2·8 mole iron and no acid-labile sulphur per mole of protein. Immunological cross-reactivity was observed between hydroxylase from strain CRL 26 and hydroxylases from other methanotrophs.

Component C

Purified component C (flavoprotein) has a molecular weight of 40 000 daltons and contains one FAD and one Fe_2S_2 per mole of protein. The purified component C from strain CRL 26 has properties similar to those of the flavoprotein component from the Bath strain (Colby and Dalton, 1978, 1979). Immunological cross-reactivity was observed between flavoprotein from strain CRL 26 and flavoproteins from other methane-utilizing organisms.

The three purified MMO systems described above involve both obligate and facultative methanotrophs; they also represent MMO systems from organisms with type I and type II internal membrane structures. The MMO systems of particulate and soluble origin are quite different. However, strong similarities in physicochemical properties have been observed in flavoprotein and hydroxylase fractions of MMO systems from the obligate *Methylococcus capsulatus* Bath and the facultative *Methylobacterium* sp. CRL 26. However, their differences in immunological properties and catalytic properties (two components vs three components, as well as specific activity) may indicate the utilization of different control mechanisms.

Potential applications

For many years, the favoured primary industrial application of methanotrophs was the production of single-cell protein (SCP). Imperial Chemical Industries, Shell and British Petroleum all considered SCP processes based

on methane as the substrate. However, most companies have shifted their interest to methanol as the substrate because methane gives mass transfer problems in an aqueous system and creates explosion hazards. For details, see the reviews by Powell and Rodgers (1984) and Vasey and Powell (1984). The production of metabolites, particularly amino acids, is another interesting area of application for methanotrophs; methanol, again, is preferred to methane for the same reasons (Morinaga and Hirose, 1984; Tani, 1985).

Since the independent discovery by several groups (Higgins *et al.*, 1979; Hou *et al.*, 1979; Dalton, 1980), that whole-cell suspensions of methanotrophs convert alkenes, alkanes and aromatic compounds to their corresponding oxygenated products, there have been intensive research activities in this area in the industrial and the academic world. Although the conversion of hydrocarbons by cell-free methanotroph systems was reported previously (Colby, Stirling and Dalton, 1977), whole-cell systems have been considered to have more potential for industrial application. The production of epoxides and of alcohols has been studied intensively.

PRODUCTION OF EPOXIDES

Resting cell suspensions of methane-grown cells of all strains tested oxidized gaseous alkenes to their corresponding epoxides, which accumulated extracellularly (*Table 3*). The optimum pH and temperature for epoxide production were pH 6·0–7·0 and approximately 35°C. At a reaction temperature of 40°C, there was an apparent decrease in the amount of epoxide accumulation. This is possibly a result both of instability of the MMO system at the higher temperature, and of the volatility of the product propylene oxide (b.p. 35°C). The reaction rate depends on the solubility of both substrates, propylene and oxygen. Substrate specificity studies for the epoxidation of 1-alkenes showed that the highest reactivity was for propylene to propylene oxide (*Table 4*). In whole-cell systems, the epoxidation of alkenes and the hydroxylation of alkanes do not require an exogeneous supply of cofactor, NADH (Hou *et al.*, 1979). The stimulation of epoxidation by methane metabolites in resting cell suspensions of methanotrophs has been reported (Hou *et al.*, 1980). These findings led these authors to a new design of bioreactor for spent biocatalyst (whole cells) regeneration.

A gas–solid bioreactor was constructed recently to produce propylene oxide from propylene using immobilized whole cells of methylotrophs (Hou, 1984b). Cell pastes of methane-grown *Methylosinus* sp. CRL 31, were physically presented as thin layers on glass beads which were packed in a glass reactor. The bioreactor was equipped with a device to control the reaction temperature. A mixture of gaseous substrates (propylene and oxygen 1 : 1 v/v) was introduced through a water bottle to pick up moisture and then into the bottom of the gas–solid bioreactor. The temperature of the bioreactor was kept at 40°C (above the boiling point of the propylene oxide, 35°C) so that the product could be collected from the exhaust gas stream by cooling. The production of propylene oxide was found to proceed at a constant rate of 18 μmol/h (*Figure 5*); the conversion of propylene was 2·7%. The

Table 3. Epoxidation of propylene to propylene oxide by methane-grown microbes

Organism	Epoxidation rate (μmol/h/mg protein)
Obligate, type II	
Methylosinus sp. (CRL 15)	2·2
Methylosinus trichosporium (OB3b)	1·8
Methylosinus sp. (CRL 16)	1·6
Methylosinus sporium (5)	1·0
Methylocystis sp. (CRL 18)	0·7
Methylocystis parvus (OBBP)	0·8
Obligate, type I	
Methylosinus sp. (CRL 4)	1·0
Methylomonas sp. (CRL 21)	1·2
Methylomonas methanica (S_1)	1·3
Methylomonas sp. (CRL 8)	0·6
Methylomonas albus (BG 8)	0·7
Methylomonas sp. (CR17)	0·5
Methylomonas sp. (CR 22)	0·8
Methylomonas sp. (CRL M6P)	0·5
Methylomonas sp. (CR 20)	1·0
Methylomonas sp. (CRL 10)	0·7
Methylobacter sp. (CRL M6)	0·6
Methylobacter sp. (CRL 23)	1·2
Methylobacter sp. (CRL M1)	2·5
Methylococcus capsulatus (Y)	0·7
Methylococcus sp. (CRL 25)	0·6
Methylococcus sp. (CRL 24)	0·9
Facultative	
Methylobacterium organophilum (CRL 26)	1·3
Methylobacterium organophilum (XX)	0·9

biocatalyst required regeneration of reducing activity after 10 hours of operation: this could be achieved *in situ* by using methanol vapour as the regenerating substrate. The biocatalyst resumed catalytic activity after the regeneration (*Figure 5*). Although the reaction conditions for this gas–solid bioreactor were not optimized with a particular strain of methanotroph, it demonstrates the feasibility of using this new type of bioreactor for the production of chemicals from gaseous substrates.

A high rate of propylene oxide production from propylene by cells of methanotrophs has been reported recently (Subramanian, 1985). Methane-grown cells of *Methylococcus capsulatus* and *Methylosinus trichosporium* oxidized propylene and 1-butene to propylene oxide and 1,2-epoxybutane, respectively, at a rate of 0·8–1·8 × 10^3 μmol/min/g dry cell mass. Whole cells were confirmed to have considerable levels of reserve reducing equivalents to catalyse the epoxidation of gaseous olefins. Methanol or formaldehyde served to regenerate the reducing equivalents. The chirality of the product 1,2-epoxybutane was determined and equal amounts of both isomers were found.

Table 4. Oxidation of alkenes and methane by resting-cell suspensions of methane-grown bacteria*

Organism	Methane to methanol	Ethylene to ethylene oxide	Propylene to propylene oxide	1-Butene to 1,2-epoxy butane	Butadiene to 1,2-epoxy butene	Isobutene to 1,2-epoxy isobutane
Obligate						
Type II membrane structure, *Methylosinus trichosporium* OB3b;	1·6	1·9	3·6	0·45	2·5	0·32
Type I membrane structure, *Methylococcus capsulatus* CRL M1 NRRL B11219	2·5	5·5	5·5	1·3	4·4	0·46
Facultative						
Methylobacterium organophilum CRL 26 NRRL B-11222	0·7	0·9	2·5	0·9	2·8	0·25

*In μmol products/h/2 mg cells (dry weight)

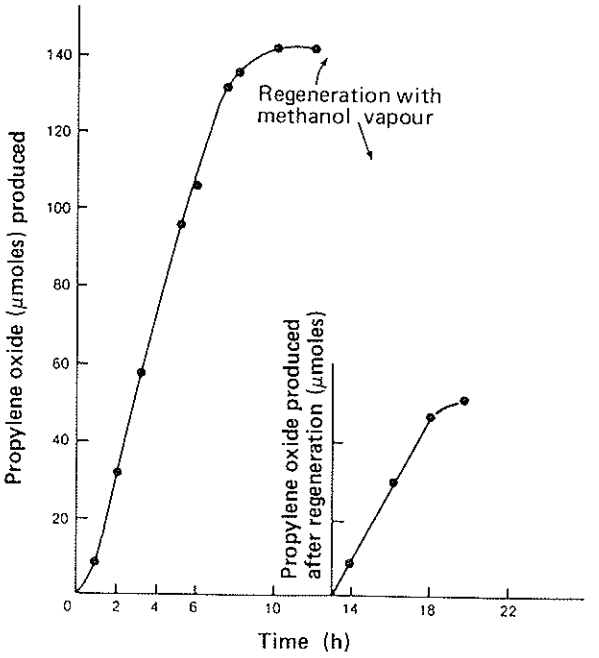


Figure 5. Production of propylene oxide from propylene by cells of methanotrophs with a gas/solid bioreactor. (From Hou, 1984b, with permission).

PRODUCTION OF ALCOHOLS

The production of methanol from methane is of great industrial interest. Although some oxidation of methane to carbon dioxide through methanol was found with cells of *Nitrosomonas* (Hyman and Wood, 1983; Jones and Morita, 1983) the cells of methanotrophs catalyse this reaction at a much higher rate. However, the oxidation of methane is hard to stop at the methanol level: methane monooxygenase and methanol dehydrogenase in the cells oxidize methanol further to formaldehyde. No enzyme inhibitor has been found to stop further utilization of the methanol produced by the cells. The use of purified methane monoxygenase to produce methanol, a bulk chemical, thus seems not to be economically feasible.

The production of primary and secondary alcohols, methyl ketones and oxygenated aromatic compounds, by methanotrophs or enzymes derived from them, have been reported (Colby, Stirling and Dalton, 1977; Higgins *et al.*, 1979; Hou *et al.*, 1979, 1981; Patel *et al.*, 1979). However, there is no large-scale development in these areas.

Conclusion

Microbial methane oxidation is a major research area from the point of view of both basic and applied science. Despite many discrepancies between the

results reported, the momentum to elucidate the mystery of microbial methane oxidation is accelerating. I believe that the chemistry of enzymatic methane oxidation will soon be understood.

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