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MINI-REVIEW

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Molecular biology and regulation of methane monooxygenase

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Abstract Methanotrophs are ubiquitous in the environment and play an important role in mitigating global warming due to methane. They are also potentially interesting for industrial applications such as production of bulk chemicals or bioremediation. The first step in the oxidation of methane is the conversion to methanol by methane monooxygenase, the key enzyme, which exists in two forms: the cytoplasmic, soluble methane monooxygenase (sMMO) and the membrane-bound, particulate methane monooxygenase (pMMO). This paper reviews the biochemistry and molecular biology of both forms of MMO. In the past few years there have been many exciting new findings. sMMO components have been expressed in heterologous and homologous hosts. The pMMO has been purified and biochemically studied in some detail and the genes encoding the pMMO have been sequenced. Copper ions have been shown to play a key role in regulating the expression of both MMO enzyme complexes. We also present a model for copper regulation based on results from Northern analysis, primer-extensions and new sequence data, and raise a number of unanswered questions for future studies.

Key words Methanotroph · Methane oxidation · Methane monooxygenase genes · Regulation

Introduction

Methane-oxidising bacteria (methanotrophs) are a unique group of gram-negative bacteria that grow aerobically on methane as sole source of carbon and energy (Hanson and Hanson 1996). Some methanotrophs also grow on methanol but none have been shown to grow on multi-carbon compounds. There has been considerable interest in methanotrophs over the past 30 years since they can be used to produce single-cell protein and bulk chemicals such as propylene oxide. The ability of these bacteria to co-oxidise a wide range of alkanes, alkenes and substituted aliphatic compounds has also been exploited in bioremediation processes, for example the degradation in soil and groundwater of key pollutants such as trichloroethylene (reviewed in Sullivan et al. 1998). Methanotrophs play an important role in the global methane cycle by oxidising methane released by methanogens in freshwater sediments and wetlands. It is also clear that methane-oxidising bacteria with a high affinity for methane can oxidise atmospheric concentrations of methane (e.g. 1–2 ppm) thereby mitigating global warming due to the effects of this greenhouse gas (Holmes et al. 1999; Dunfield et al. 1999).

Techniques for the enrichment and isolation of methanotrophs from soils, freshwater and sediments (Whittenbury et al. 1970) facilitated the isolation of over 100 methanotroph strains. These were grouped into five proposed genera, based on their morphology, intracytoplasmic membranes, type of resting stage formed and other physiological and morphological traits. This classification scheme has not changed significantly. Methanotrophs are divided into two distinct groups. Type I methanotrophs are γ-Proteobacteria of the genera *Methylomonas*, *Methylobacter*, *Methylomicrobium*, *Methylococcus*, *Methylocaldum* and *Methylosphaera*. These bacteria possess bundles of intracytoplasmic membranes, contain predominantly 16-carbon fatty acids and assimilate formaldehyde produced from the oxidation of methane (via methanol) by the ribulose monophosphate cycle. Type II methanotrophs of the genera *Methylosinus* and *Methylocystis* are α-Proteobacteria, have intracytoplasmic membranes arranged around the periphery of the cell, contain predominantly 18-carbon fatty acids in their membranes and assimilate formaldehyde into cell carbon using the serine cycle (Hanson and Hanson 1996). Recently it has been shown that methanotrophs also have a tetrahydromethanopterin-based C_1 metabolism. Therefore the question of how methanotrophs metabolise methanol generated by methane monooxygenase has to be readdressed (Vorholt et al. 1999). The ma-

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jority of extant methanotrophs are mesophiles isolated from near neutral pH environments. However, more recently, methanotrophs have been isolated from more extreme environments such as hot springs (Bodrossy et al. 1995), acidic peat bogs (Dedysh et al. 1998), soda lakes (Khmelenina et al. 1997) and the Antarctic (Bowman et al. 1997), indicating that these bacteria are ubiquitous in the natural environment.

The first enzyme in the methane oxidation pathway, is methane monooxygenase (MMO). There are two distinct types of MMO enzymes: a soluble, cytoplasmic enzyme complex (sMMO) and a membrane-bound, particulate enzyme system (pMMO).

Soluble methane monooxygenase

Not all methanotrophs possess sMMO. Until recently, it was thought that this enzyme was confined to the type II genera *Methylosinus* and *Methylocystis* and the type I methanotroph *Methylococcus capsulatus* (Bath). However, sMMO has now been characterised from one marine *Methylomicrobium* (Fuse et al. 1998) and one *Methylomonas* (Shigematsu et al. 1999) species. In contrast to pMMO, sMMO has extremely broad substrate specificity and can oxidise a wide range of non-growth substrates such as alkanes, alkenes and aromatic compounds thus making it the more attractive enzyme for co-oxidation reactions and bioremediation processes (Sullivan et al. 1998). sMMO is expressed only under conditions in which the copper-to-biomass ratio is low, i.e. under "low-copper" growth conditions, when copper ions are omitted from the trace elements solution of a standard mineral salts medium or cells are grown in a fermentor to high cell densities (OD>6.0; see below). There is also some evidence that copper ions inhibit the activity of sMMO (Jahng and Wood 1996).

Biochemistry of sMMO

The most extensively characterised sMMO enzymes are those from *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b (reviewed in Lipscomb 1994; Deeth and Dalton 1998). The sMMO is a non-haem iron-containing enzyme complex consisting of three components. The hydroxylase consists of three subunits of 60, 45 and 20 kDa arranged in an $\alpha_2\beta_2\gamma_2$ configuration. The α-subunit contains a non-haem bis-μ-hydroxo-bridged binuclear iron centre where methane and oxygen interact to form methanol at the active site of the enzyme. The crystal structures of the hydroxylase components from *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b have been reported (Rosenzweig et al. 1993; Lipscomb 1994; Elango et al. 1997). The di-iron centre of the hydroxylase resides approximately 12 Å below the floor of two canyon regions formed by its α- and β-subunits. Like many other multi-component oxygenase systems, sMMO contains a component of approximately

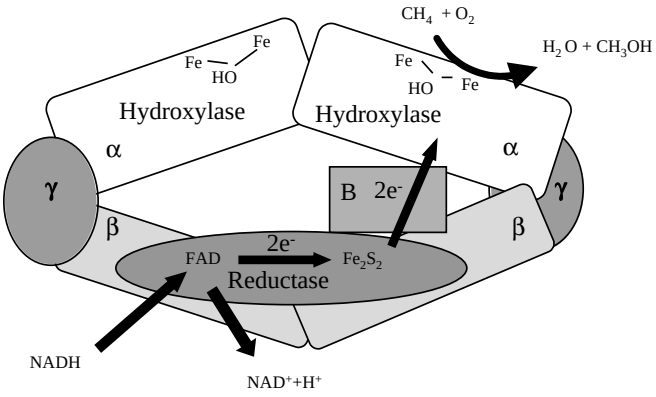

Fig. 1 Model (not to scale) of the soluble methane monooxygenase complex from *Methylococcus capsulatus* (Bath) showing the proposed pathway of electron flow from NADH to the di-iron centre and active site of the α-subunit of the hydroxylase component

16 kDa, Protein B, which serves an "effector" or regulatory role. The activity of Protein B may be regulated by proteolysis at its amino terminus (Lloyd et al. 1997). At low concentrations, Protein B converts the hydroxylase from an oxidase to a hydroxylase and stabilises intermediates necessary for oxygen activation. Saturating amounts of Protein B dramatically increase the rates of formation of intermediates and accelerate catalysis of methane to methanol by sMMO (Lee and Lipscomb 1999). The structure of Protein B from *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* (Bath) has recently been obtained by NMR spectroscopy (Walters et al. 1999). The third component, Protein C, is a 39 kDa NADH-dependent, [2Fe-2S]- and FAD-containing reductase that accepts electrons from $NADH_2$ and transfers them to the di-iron sites of the hydroxylase (Lund et al. 1985; see Fig. 1).

Molecular biology of sMMO

The genes encoding sMMO from several methanotrophs have now been cloned and sequenced. The most extensively studied are those from *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b (Murrell 1994). sMMO genes are clustered on the chromosome of these methanotrophs (Fig. 2a). *mmoX*, *mmoY* and *mmoZ* encode the α-, β- and γ-subunits respectively of the hydroxylase. *mmoB* and *mmoC* code for Protein B and the reductase component. Interestingly, *mmoB* lies between *mmoY* and *mmoZ*; an ORF of unknown function, designated *orfY*, with a coding capacity of 12 kDa, lies between *mmoZ* and *mmoC* in all genes clusters analysed to date (McDonald et al. 1997). We have expressed *orfY* from *Methylococcus capsulatus* (Bath) as a glutathione S-transferase fusion polypeptide, purified it by affinity chromatography, raised antibodies to this polypeptide and then probed cell-free extracts of *Methylococcus capsulatus* (Bath) grown under a variety of different conditions including high- and low-copper media (see later). However, no cross-reactivity was observed with this antibody and

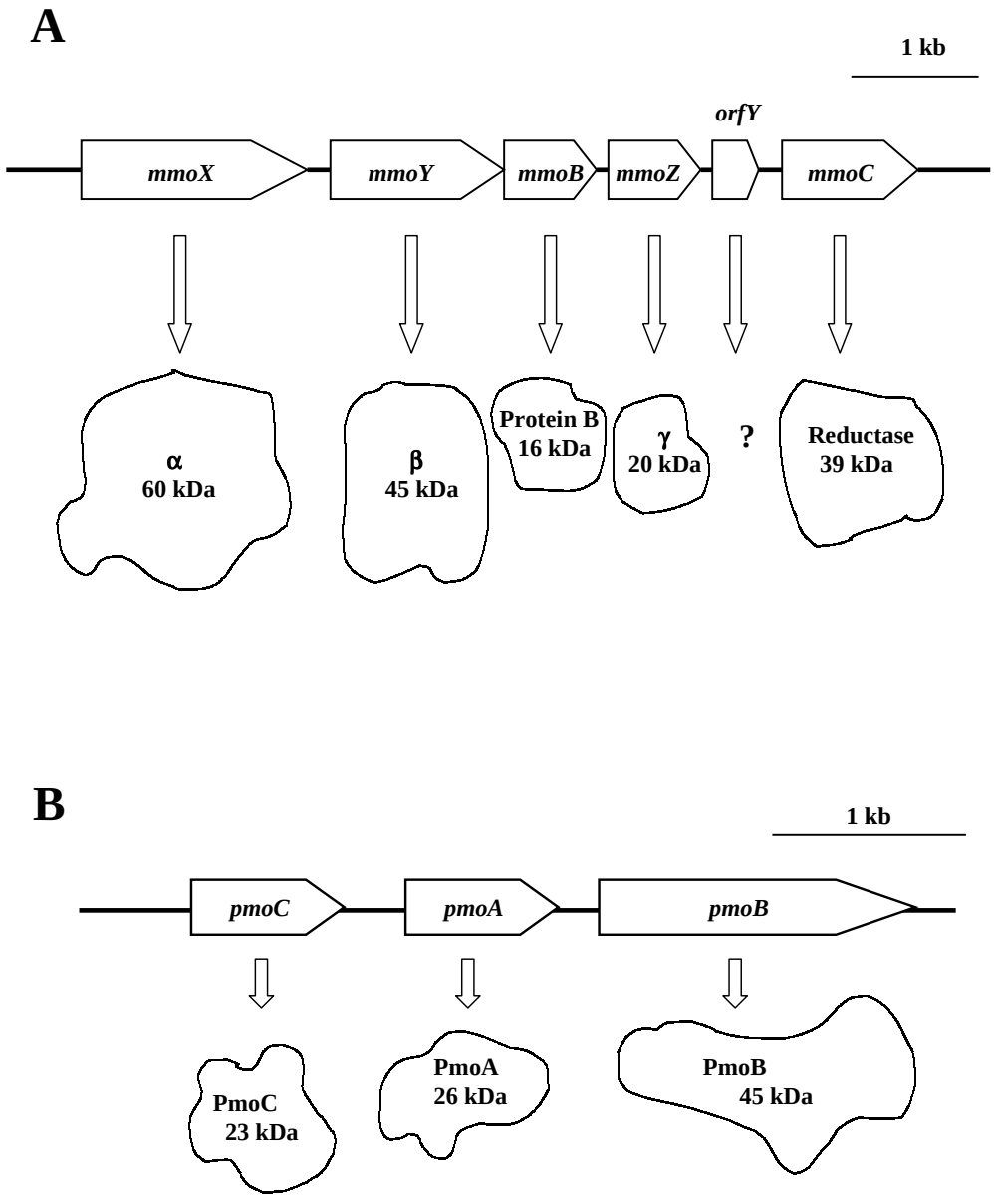

Fig. 2 The soluble methane monooxygenase (**A**) and particulate methane monooxygenase (**B**) gene clusters of methane-oxidising bacteria

polypeptides from *Methylococcus capsulatus* (Bath) and therefore the function (if any) of *orfY* remains unclear (Lloyd, Dalton and Murrell, unpublished data).

The sMMO genes are highly conserved in all genera of methanotrophs so far studied, nucleotide sequences are 55–94% identical and amino acid sequences are 47–96% identical. Upstream of the sMMO gene cluster of *Methylosinus trichosporium* OB3b lie genes encoding the chaperones Hsp60 and Hsp10; however their role, if any, in the activity of sMMO is not known (McDonald and Murrell, unpublished data).

Expression of sMMO genes

Protein B and the reductase component of sMMO from *Methylococcus capsulatus* (Bath) were active when expressed in *Escherichia coli* using a T7 polymerase expression system but the recombinant hydroxylase was inactive (West et al. 1992). This was thought to be due to the lack of necessary "assembly factors" in this heterologous host. However, the ability to express an active Protein B in *E. coli* enabled us to carry out the first site-directed mutagenesis experiments on the sMMO. Protein B in *Methylococcus capsulatus* (Bath) is subject to N-terminal cleavage between specific amino-acyl residues, Met-12 and Gly-13, which results in inactivation of this "effector/regulatory" polypeptide (Gallagher et al. 1999). This N-terminal cleavage does not appear to occur with Protein B from *Methylosinus trichosporium* OB3b, where the N-terminal sequence contains Met-12 and Gln-13. The substitution of Gly-13 with Gln-13 in the recombinant Protein B of *Methylococcus capsulatus* (Bath) dramatically stabilised the activity of this polypeptide (Lloyd et al. 1997).

Subsequently, further mutations of Protein B have been constructed; analysis of the mutant proteins confirms that the amino terminus of Protein B is very sensitive to hydrolysis (Brandstetter et al. 1999).

The expression of active recombinant sMMO from *Methylosinus trichosporium* OB3b was reported in *Pseudomonas putida* F1, *Agrobacterium tumefaciens* and *Rhizobium meliloti* (Jahng et al. 1996). However, when recombinant strains were assayed using the standard MMO assay, by following the conversion of propylene to propylene oxide, negligible sMMO activity was obtained (Jahng et al. 1996; Lloyd, Dalton and Murrell, unpublished data). Experiments with *E. coli* demonstrated that recombinant *Methylosinus trichosporium* OB3b sMMO hydroxylase polypeptides were expressed, albeit at low levels; however the hydroxylase was inactive, suggesting that it was not assembled correctly in *E. coli* (Lloyd, Dalton and Murrell, unpublished data).

Heterologous expression of sMMO from *Methylosinus trichosporium* OB3b has been achieved using the methanotrophs *Methylomicrobium album* BG8 and *Methylocystis parvus* OBBP. Neither of these strains possesses sMMO. They were thought to be potentially better hosts for expression studies since they are more closely related to *Methylosinus trichosporium* and may contain the necessary proteins for the correct assembly and/or insertion of the di-iron centre into the hydroxylase of sMMO. Expression was achieved by conjugating a broad-host-range plasmid containing the native promoter and sMMO gene cluster from *Methylosinus trichosporium* OB3b into *Methylocystis parvus* OBBP and *Methylomicrobium album* BG8. These strains, when harbouring this plasmid, expressed active sMMO. *Methylomicrobium album* BG8 constitutively expressed pMMO when grown at both high and low copper-to-biomass ratios; however, transcription of the recombinant sMMO genes was only observed under growth conditions of low copper-to-biomass ratios (see later). Therefore, either this heterologous host contains a regulatory system for sMMO or the regulatory polypeptide(s) necessary for sMMO expression is contained on this plasmid (Lloyd et al. 1999a).

Homologous expression of the sMMO gene cluster from *Methylosinus trichosporium* OB3b has been achieved using a sMMO-minus mutant of this organism. Although it is very difficult to obtain mutants of methanotrophs, marker-exchange mutagenesis has been used to generate a mutant *Methylosinus trichosporium* OB3b with a kanamycin cassette inserted into the chromosomal gene *mmoX* (Martin and Murrell 1995). This mutant strain exhibits a sMMO-minus, pMMO-positive phenotype. Complementation of the sMMO-minus phenotype was achieved by conjugating into this mutant the broad-host-range recombinant plasmid pVK100Sc, which contains the native promoter and sMMO operon from *Methylosinus trichosporium* OB3b. The sMMO-minus mutant containing pVK100Sc displayed a sMMO-positive phenotype. In wild-type *Methylosinus trichosporium* OB3b, copper ion concentrations greater than about 0.25 μM repress expression of sMMO genes. The stable maintenance of pVK100Sc in the sMMO-minus mutant resulted in expression of sMMO in this recombinant strain at copper concentrations of 7.5 μM. However, active sMMO was only detected in cell-free extracts from this strain when grown with 7.5 μM copper ions after the addition of excess purified sMMO reductase component, because the reductase is inhibited by copper ions in vitro. This expression of sMMO at elevated copper concentrations may have been due to the increased copy number of sMMO genes carried on pVK100Sc. To date, this is the only report of expression of recombinant sMMO at specific activities that are at least as high as in the wild-type organism. Indeed, under low-copper growth conditions, the specific activity of sMMO in the recombinant *Methylosinus trichosporium* was approximately three times higher than in the wild-type organism (Lloyd et al. 1999b). This now allows the possibility for carrying out site-directed mutagenesis and structure–function studies on the hydroxylase of sMMO, and we are currently investigating the effects of mutating key residues in the α-subunit of the hydroxylase.

Particulate methane monooxygenase

Virtually all methanotrophs studied to date possess pMMO. The only exception to this appears to be an acidophilic methanotroph, strain K, isolated from northern wetland peat, which is a member of the α subclass of Proteobacteria and most closely related to *Beijerinckia indica* (Dedysh et al. 1998). This strain has been shown to contain a sMMO similar to those of type II methanotrophs but does not appear to contain pMMO (Dedysh and Semrau, personal communication). The pMMO in methanotrophs is expressed under growth conditions in which the copper-to-biomass ratio is high. Removing all trace amounts of copper ions from the growth medium results in poor growth of methanotrophs such as *Methylomicrobium album* BG8 or *Methylocystis parvus* OBBP which do not possess sMMO.

Biochemistry of pMMO

The first partial purification of pMMO from *Methylococcus capsulatus* (Bath) used dodecyl β-D maltoside to solubilise active fractions from the membranes of this organism grown under "high-copper" growth conditions (Smith and Dalton 1989). Subsequent studies have concentrated on the pMMO from this strain, although recently, pMMO from *Methylosinus trichosporium* OB3b has been reported (Takeguchi et al. 1998).

The pMMO consists of three subunits of approximately 45, 27 and 23 kDa (Zahn and DiSpirito 1996; Nguyen et al. 1998) in a stoichiometry of 1:1:1. The 45 and 27 kDa polypeptides probably contain the active site because they are specifically labelled by [^{14}C]-acetylene, a suicide substrate for MMO (Prior and Dalton 1985; Zahn and DiSpirito 1996). The active enzyme contains 2 iron and approximately 15 copper atoms per mol. Chan and co-workers

have proposed that trinuclear copper clusters are involved in catalysis (Nguyen et al. 1996), whereas DiSpirito and colleagues (Tellez et al. 1998) suggest that the catalytic site involves both iron and copper atoms. In addition, small copper-binding polypeptides of 1,218 and 779 Da are associated with the pMMO. These copper-binding compounds isolated from the spent medium of *Methylosinus trichosporium* OB3b (Tellez et al. 1998) have a high affinity for copper ions. Their spectral and other characteristics have been examined in detail (DiSpirito et al. 1998). In wild-type strains, the concentration of copper-binding compounds in the culture medium was highest when cells were expressing pMMO but had used the majority of copper ions from the growth medium. In sMMO-constitutive mutants of *Methylosinus trichosporium* OB3b (Tellez et al. 1998) high concentrations of copper-binding compounds were present during growth on high- and low-copper medium (DiSpirito et al. 1998). These copper-binding compounds appear to bind a large proportion of the copper associated with the membrane fraction in cells expressing pMMO. They may be important for sequestering copper ions, stabilising the pMMO and/or maintaining the appropriate redox state for the pMMO (discussed in DiSpirito et al. 1998). In vitro, both duroquinone and $NADH_2$ can be used as reductant for pMMO but the physiological reductant for pMMO is not known. Potential in vivo reductants could include cytochromes $b_{559/569}$, *c* or $c_{553.}$ Unlike the sMMO, the pMMO has relatively narrow substrate specificity, oxidising alkanes and alkenes of up to five carbons but not aromatic compounds.

Molecular biology of pMMO

The genes encoding pMMO from *Methylococcus capsulatus* (Bath) have been cloned and sequenced (Semrau et al. 1995; Stolyar et al. 1999) and are clustered on the chromosome in the order *pmoCAB* (Fig. 2b). There are two virtually identical copies of these genes (13 bp changes over 3,183 bp of *pmoCAB*) present in the genome of *Methylococcus capsulatus* (Bath) and a third copy of *pmoC* has also been identified (Stolyar et al. 1999). This is very similar to the analogous system in nitrifiers that also contain two copies of genes encoding ammonia monooxygenase, *amoCAB*, and a third *amoC* gene (Sayavedra-Soto et al. 1998).

Comparison of *pmo* and *amo* genes from methanotrophs and nitrifiers suggests that the pMMO and AMO may be evolutionarily related (Holmes et al. 1995; Klotz and Norton 1998). It is not clear why multiple copies of these genes are present in such bacteria. In *Nitrosomonas europaea*, an insertion mutant defective in one *amoA* gene exhibited slower growth than the wild-type, while a mutant in copy 2 of *amoA* showed normal growth. Lidstrom and colleagues (Stolyar et al. 1999) have constructed chromosomal insertion mutants in all seven *pmo* genes in *Methylococcus capsulatus* (Bath). With the exception of the lone third copy of *pmoC*, for which no null mutants could be obtained, all other mutants grew on methane, suggesting that both sets of genes were functionally equivalent. Copy 1 mutants showed about two-thirds of the wild-type methane oxidation activity while copy 2 mutants had about one-third of the activity of the wild type strain. No double null mutants defective in both copies of *pmoCAB* were obtained which suggests that the cells require pMMO for normal growth (Stolyar et al. 1999).

The entire pMMO gene clusters from two more genera of methanotrophs, *Methylosinus trichosporium* OB3b and *Methylocystis* sp. strain M, have recently been cloned and sequenced (Gilbert, McDonald and Murrell, unpublished data). As with all other methanotrophs studied to date (Semrau et al. 1995), they also have two copies of *pmoCAB*. Comparative analysis of derived pMMO and AMO polypeptide sequences highlight the similarities between these two enzyme systems and the conserved nature of their sequences (42–87% identity and 58–95% similarity at the amino acid level). PmoC and PmoA are predicted to be highly hydrophobic and consist mainly of putative transmembrane-spanning helices, whereas PmoB only has two putative transmembrane regions. It has only been possible to clone these *pmo* gene clusters on overlapping DNA fragments, since parts of these genes appear to be toxic to the *E. coli* host; therefore expression of pMMO in heterologous hosts may be difficult, if not impossible. We are currently expressing *pmoA* gene/glutathione S-transferase fusions in *E. coli* in order to raise antibody to pMMO for immunogold labelling experiments designed to localise pMMO on the membranes of methanotrophs (Gilbert and Murrell, unpublished data).

The availability of a large dataset of pMMO gene sequences from methanotrophs allows the use of *pmo* as a "functional gene probe" in molecular ecology experiments in order to examine the diversity of methanotrophs in the natural environment (recently reviewed in Murrell et al. 1998; Costello and Lidstrom, 1999; Dunfield et al. 1999; Henckel et al. 1999; Holmes et al. 1999).

Regulation of MMO

In methanotrophs such as *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* (Bath), which possess both sMMO and pMMO enzyme systems, a unique metabolic switch mediated by copper ions occurs. In cells grown under high copper-to-biomass ratios, the pMMO enzyme is expressed and there is no detectable sMMO expression. However, when cells are essentially starved for copper and the copper-to-biomass ratio is low (<0.2 μM Cu^{2+} for a typical flask batch culture of *Methylosinus trichosporium* OB3b), then the sMMO enzyme is expressed. This phenomenon, the "copper switch", is mediated exclusively by copper ions (Stanley et al. 1983). Copper ions irreversibly inhibit the activity of sMMO in vivo and in vitro by inactivating the reductase component (Jahng and Wood 1996).

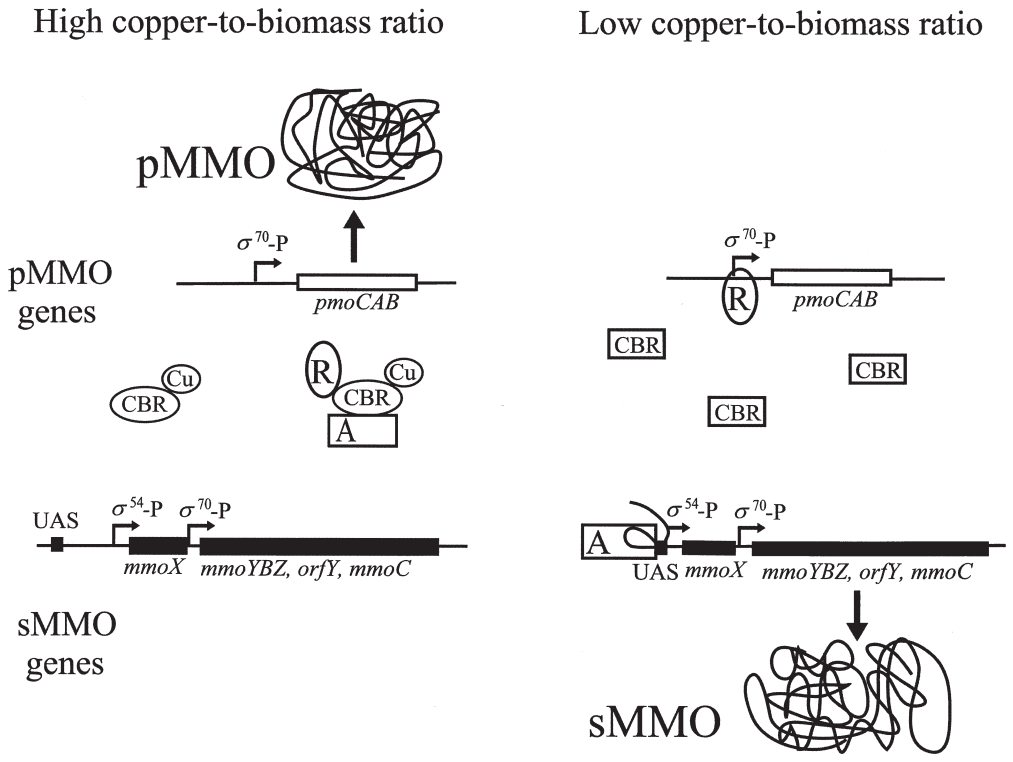

Fig. 3 Model for the copper regulation of the genes encoding the methane monooxygenase in *Methylosinus trichosporium* OB3b. Under high copper-to-biomass ratio, the pMMO enzyme complex is expressed, whereas under low copper-to-biomass ratio, the sMMO is expressed. The regulators repressor (*R*), activator (*A*), and copper-binding regulator (*CBR*) are all hypothetical. The σ^{70} promoter of the *pmo* operon is also hypothetical, but in the closely related *Methylocystis* sp. strain M, the putative promoter is a σ^{70} promoter. When the CBR binds copper under high copper-to-biomass ratios, its conformation changes and it binds to R and to A, thus preventing R from repressing the *pmo* genes and A from activating the *smo* genes. However, under low copper-to-biomass ratios, the CBR cannot form a complex with R and/or A. So, R represses the transcription of the *pmo* genes and A activates the *smo* genes by binding to the hypothetical UAS sequence and enabling σ^{54}-associated RNA polymerase to initiate transcription. Alternatively, copper binding could change directly the conformation of R and A, thereby changing their affinity to putative operator sequences

Regulation of expression of sMMO

Transcription of sMMO is arrested in cells exposed to high levels of copper ions and sMMO mRNA transcripts were not detected 15 min after the addition of $CuSO_4$ (Nielsen et al. 1996). Northern blotting and primer-extension experiments with *Methylococcus capsulatus* (Bath) revealed that the six ORFs of the sMMO gene cluster are organised as an operon. Three transcripts were identified, one of which (approximately 5.5 kb and faintly detectable) may be the full-length transcript encoding the gene products of the entire sMMO gene cluster. The only putative promoter identified was one showing rather weak identity to *E. coli* –35, –10 consensus sequences, located upstream of the *mmoX* gene and the only primer-extension product obtained was 37 bp upstream of the *mmoX* start codon (Nielsen et al. 1996).

Northern analysis and primer extension experiments with *Methylosinus trichosporium* OB3b also showed that transcription of sMMO genes is switched off within about 10 min after the addition of 50 μM copper sulfate to a steady-state chemostat culture expressing sMMO. Three major sMMO gene cluster transcripts (1.8, 2.2 and 4.0 kb) were identified and were presumed to correspond to transcripts of: (1) *mmoX*; (2) *mmoY, mmoB, mmoZ* and (3) *mmoY, mmoB, mmoZ, orfY* and *mmoC*. Occasionally a full-length transcript of 5.5 kb encoding the entire sMMO gene cluster could be observed but this was clearly unstable (Nielsen et al. 1997). Primer-extension analysis of sMMO from *Methylosinus trichosporium* OB3b showed that transcription of the *mmoX* gene is directed from a promoter immediately upstream from the transcription initiation site which has a sequence very close to the consensus sequence recognised by *E. coli* RNA polymerase containing σ^{54}. Further primer-extension experiments revealed the presence of a second transcript initiating between *mmoX* and *mmoY*, upstream of which are putative –35, -10 sequences similar to those of σ^{70}-like promoters. Transcription at this putative promoter also appears to be repressed by copper ions although the mechanism by which this occurs is unclear at present (Nielsen et al. 1997). Consistent with the loss of sMMO activity and repression of sMMO expression during the copper switch experiment was the appearance of *pmo* transcripts of approximately 4.0 and 1.2 kb.

Similar transcript analysis has been carried out with *pmo* gene clusters from *Methylosinus trichosporium* OB3b and *Methylocystis* sp. strain M, in which the

pmoCAB clusters are probably transcribed from a single transcriptional start site located 300 bp upstream of the start of the first gene *pmoC*, initiating at a putative σ^{70} promoter which is negatively regulated under low-copper conditions (Gilbert et al. 2000).

There are a number of features of the reciprocal regulation of the sMMO and pMMO gene clusters in methanotrophs that remain unclear at present. Copper ions may mediate transcription of sMMO gene promoters via a regulator protein which, upon binding this metal, acquires the ability to bind to operator regions on the promoter, i.e. the classical repressor model whereby a repressor protein prevents transcriptional initiation by interfering with the RNA polymerase or shielding promoter sequences. An alternative model is one in which expression of sMMO genes is subject to positive control, with copper ions binding to and inactivating an activator protein (Fig. 3). The second alternative is supported by the presence of a σ^{54} promoter upstream of *mmoX* in *Methylosinus trichosporium* OB3b, since all known σ^{54} promoters depend on activator proteins for the formation of a transcription-competent open complex. We are currently cloning *rpoN* encoding σ^{54} from *Methylosinus trichosporium* OB3b in order to mutate this gene and then examine the effects on sMMO expression in this methanotroph (Stafford and Murrell, unpublished data). Activation of *pmo* genes probably involves a copper-binding activator protein; however, an alternative hypothesis (Fig. 3) is that these genes may be subject to negative control in the absence of copper ions. A hypothetical model for the copper-dependent transcriptional regulation of MMO genes is given in Fig. 3.

Unanswered questions and future research

There are still a number of intriguing questions about methanotrophs and their key enzyme MMO. Firstly, why do only some methanotrophs contain sMMO and what advantage if any does this confer on methanotrophs? There may be some competitive advantage for a methanotroph to use sMMO in environments where copper is depleted, for example peat bogs where there may be a relatively high number of methanotrophs and little bioavailable copper. This may be why methanotrophs containing sMMO appear to predominate in this environment (McDonald et al. 1996; Dedysh et al. 1998). The evolutionary link between pMMO and AMO and why there are two sets of genes for these enzymes needs to be explored, as do their precise location within the cell. The assembly of sMMO and structure–function studies designed to identify key residues in the hydroxylase that confer specificity and stereoselectivity and residues important in channelling electrons to the active site are also needed. Assembly of pMMO, the mechanism of copper uptake and sequestering, types of electron donors and mechanism of methane activation will require considerable effort. The molecular mechanisms for the regulation of expression of sMMO and pMMO need to be elucidated. Finally, the genome of *Methylococcus capsulatus* is to be sequenced, which will further underpin research into regulation and proteomics of methanotrophs.

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