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Biological Methane Oxidation: Regulation, Biochemistry, and Active Site Structure of Particulate Methane Monooxygenase

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Particulate methane monooxygenase (pMMO) is a three-subunit integral membrane enzyme that catalyzes the oxidation of methane to methanol. Although pMMO is the predominant methane oxidation catalyst in nature, it has proved difficult to isolate, and most questions regarding its molecular structure, active site composition, chemical mechanism, and genetic regulation remain unanswered. Copper ions are believed to play a key role in both pMMO regulation and catalysis, and there is some evidence that the enzyme contains iron as well. A number of research groups have solubilized and purified or partially purified pMMO. These preparations have been characterized by biochemical and biophysical methods. In addition, aspects of methane monooxygenase gene regulation and copper accumulation in methanotrophs have been studied. This review summarizes for the first time the often controversial pMMO literature, focusing on recent progress and highlighting unresolved issues.

Keywords pMMO, methanotroph, copper, metalloenzyme, membrane protein

INTRODUCTION

Methane monooxygenase (MMO) enzyme systems convert methane, the most inert hydrocarbon (C–H bond energy, 104 kcal/mole), to methanol. This reaction is the first step in the metabolic pathway of methanotrophs, bacteria that utilize methane as their sole source of carbon and energy (Anthony, 1982). There are two types of MMO systems, a soluble, cytoplasmic complex (sMMO) and a membrane-bound, particulate system (pMMO). Of approximately 130 known methanotrophs (Bowman *et al.*, 1993; Hanson & Hanson, 1996), all produce pMMO, and seven strains produce both sMMO and pMMO. In these strains, sMMO is only expressed under conditions of low copper availability (Stanley *et al.*, 1983; Prior & Dalton, 1985; Murrell *et al.*, 2000b). Although pMMO is the predominant methane oxidation catalyst in nature, it has proved difficult to isolate, and most investigators have instead opted to study sMMO, which consists of three components: a hydroxylase (MMOH), a reductase (MMOR), and a regulatory protein (MMOB). The hydroxylase belongs to a family of diiron carboxylate-bridged proteins that includes the ribonucleotide reductase R2 protein and the stearoyl acyl carrier protein Δ^9 desaturase (Solomon *et al.*, 2000). The biochemistry, structure, and mechanism of sMMO are well understood and have been reviewed frequently over the last decade (Lipscomb, 1994; Liu & Lippard, 1995; Wallar & Lipscomb, 1996; Rosenzweig & Lippard, 1997; Whittington *et al.*, 1998; Merkx *et al.*, 2001; Baik *et al.*, 2003). By contrast, the details of pMMO biochemistry and function are only beginning to be unraveled and have not yet been reviewed comprehensively.

MMO systems have been the focus of intense study in recent years for several reasons. Only two enzymes are known to oxidize methane, MMO, and ammonia monooxygenase (AMO), an enzyme that oxidizes ammonia to hydroxylamine in nitrifying bacteria (Hyman *et al.*, 1988; Arp *et al.*, 2002). Knowledge of how these enzymes activate the methane C–H bond could impact development of new synthetic catalysts. Methane oxidation, accomplished by the enzymes at ambient temperature and pressure, presents a formidable problem to industry. Platinum (Periana *et al.*, 1998) and mercury (Periana *et al.*, 1993) catalysts have been developed, but require high temperatures and pressures. Improvement of these catalysts is important because current reserves of natural gas, composed of methane and hydrogen gases, are

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underutilized as an energy source due to difficulties with transport (Voss, 2002). Efficient conversion of methane to methanol would solve this problem and could have significant implications for the use of methane as an alternative energy source.

Another important property of MMOs is the ability to oxidize a variety of alkanes and alkenes in addition to methane. In particular, both sMMO and pMMO can oxidize halogenated hydrocarbons. Chlorinated hydrocarbons are the most frequently detected groundwater pollutants, with trichloroethylene (TCE), 1,2-dichloromethane, and chloroform the most abundant (Hanson & Hanson, 1996). TCE is widely used in degreasing metal parts, preparing textiles, and synthesizing polyvinyl chloride, pharmaceuticals, and insecticides, and is a suspected human carcinogen (U. S. Department of Health and Human Services, 1997). Some anaerobic bacteria convert TCE as well as dichloroethylene and tetrachloroethylene to vinyl chloride (Hanson & Hanson, 1996), another known carcinogen (Kielhorn *et al.*, 2000). Efforts to use MMO for TCE bioremediation have typically focused on sMMO (Sullivan *et al.*, 1998; van Hylckama Vlieg & Janssen, 2001). pMMO is also capable of oxidizing TCE (DiSpirito *et al.*, 1992; Lontoh & Semrau, 1998), and is more practical for *in situ* bioremediation applications since all methanotrophs produce pMMO. In addition, polluted groundwater typically contains high concentrations of copper (Phelps *et al.*, 1992), which would repress sMMO expression.

Finally, study of MMO structure and function is motivated by the link between methane and global warming. The contribution of methane as a greenhouse gas is second only to that of carbon dioxide, and methane absorbs significantly more infrared radiation (Tol *et al.*, 2003). Methane emissions due to agricultural and industrial activities have more than doubled over the past two centuries. Global warming due to increasing greenhouse gas emissions can have detrimental effects on the environment and, consequently, on human health (Epstein 2000, 2002). One way to control methane emissions is to vent landfills and oil wells through soil populated with methanotrophic bacteria (Stein & Hettiaratchi, 2001; Park *et al.*, 2002).

Whereas sMMO has been well characterized, most questions regarding the genetic regulation, molecular structure, and chemical mechanism of pMMO remain unanswered. The evidence to date points to a primary role for copper ions in pMMO regulation as well as in pMMO catalysis. Recent studies have begun to shed light on copper accumulation and gene regulation in methanotrophs. A number of researchers have reported methods to isolate, solubilize, and purify the pMMO complex. The presence of iron in the enzyme is controversial. The partially purified or purified enzyme has been analyzed by biochemical and spectroscopic techniques, and mechanistic investigations have been initiated. Here we review the often confusing and contentious pMMO literature, focusing on recent progress and emphasizing key outstanding issues.

BACKGROUND

Methanotrophic Bacteria

Methanotrophic bacteria are a unique family of gram negative aerobic eubacteria that use methane as their only source of carbon and energy (Anthony, 1982). Methanotrophs have been known since the early twentieth century, but isolation procedures were only developed by Whittenbury and coworkers in 1970 (Whittenbury *et al.*, 1970). Methanotrophs are found in mud, swamps, rivers, rice paddies, streams, oceans, ponds, meadow soils, sediments, deciduous woods, and sewage sludge (Hanson & Hanson, 1996). Strains from extreme environments have also been isolated, including acidophilic strains from peat wetlands in northern Europe and Siberia (Dedysh *et al.*, 1998), alkaliphilic strains from soda lakes in central Asia (Khmelenina *et al.*, 1997) and Kenya (Sorokin *et al.*, 2000), thermophilic strains from hot springs in Hungary (Bodrossy *et al.*, 1999), and psychrophilic strains from saline lakes in Antarctica (Bowman *et al.*, 1997). Various techniques to study methanotroph ecology have facilitated the discovery of additional strains. For example, oligonucleotide probes can be used to detect genes unique to methanotroph metabolism, such as those encoding MMO, or to detect 16S ribosomal RNA sequences specific to certain types of methanotrophs (Hanson & Hanson, 1996; Murrell *et al.*, 1998). Alternatively, signature phospholipid profiles can be obtained either by liquid chromatography/electrospray ionization/mass spectrometry analysis (Fang *et al.*, 2000) or by ^{14}C phosopholipid ester-linked fatty acid content after radiolabelling soil samples with $^{14}CH_4$ (Holmes *et al.*, 1999).

Methanotrophs are obligate C_1 metabolizers (Whittenbury & Dalton, 1981). The first step in their metabolic pathway is the oxidation of methane to methanol by MMO enzyme systems (Figure 1). Methanol is converted to formaldehyde by methanol dehydrogenase. Formaldehyde is then oxidized to formate and carbon dioxide by formaldehyde and formate dehydrogenases, respectively. Formaldehyde is also assimilated for biosynthesis of multicarbon compounds. The type I methanotrophs, including genera *Methylomonas*, *Methylobacter*, *Methylomicrobium*, *Methylosphaera*, and *Methylocaldum*, assimilate formaldehyde via the ribulose monophosphate pathway (RuMP), whereas the type II methanotrophs, such as genera *Methylosinus* and *Methylocystis*, utilize the serine pathway. The type X methanotrophs, such as *Methylococcus*, use the RuMP but also possess low levels of the

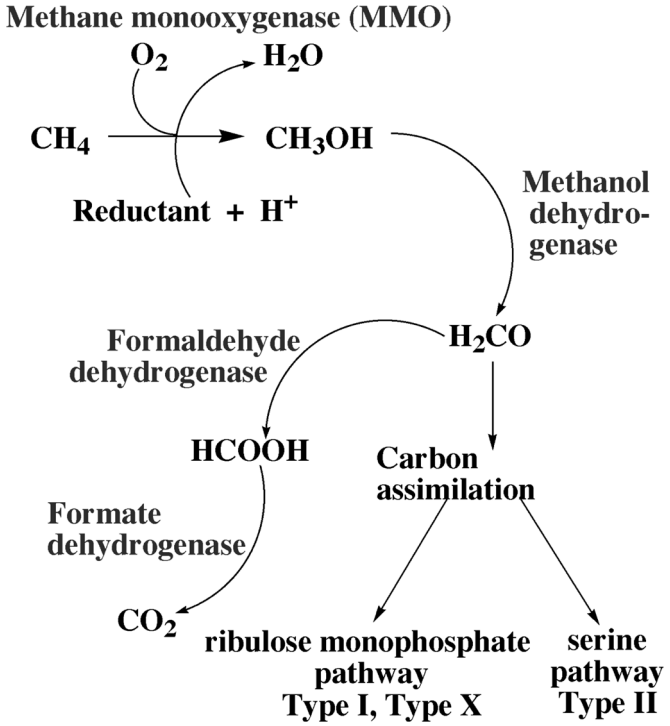

FIG. 1. Metabolic pathway of methanotrophs.

serine pathway enzyme ribulose-bisphosphate carboxylase. Type X methanotrophs grow at higher temperatures than type I or type II organisms. These and other distinguishing features, such as cell morphology and membrane arrangement, have been reviewed previously (Hanson & Hanson, 1996).

MMO Genes

The genes encoding pMMO and sMMO have been cloned and sequenced from multiple organisms (Murrell *et al.*, 2000a, 2000b) (Figure 2). The genes for pMMO (*pmo)* are organized in the *pmoCAB* operon in which *pmoB*, *pmoA*, and *pmoC* encode polypeptides corresponding to pMMO subunits α (~46 kDa), β (~28 kDa), and γ (~29 kDa), respectively (Semrau *et al.*, 1995a). These values include putative signaling sequences, resulting in typical molecular masses by SDS-PAGE of 45, 24, and 22 kDa for α, β, and γ, respectively. Using the dense alignment surface (DAS) method (Cserzö *et al.*, 1997), the α subunit is predicted to have three transmembrane domains, the β subunit five, and the γ subunit six. Two nearly identical copies of *pmoCAB* are found in *M. capsulatus* (Bath) (Semrau *et al.*, 1995a), *M. trichosporium* OB3b, and *Methylocystis* sp. strain M (Gilbert *et al.*, 2000), and a third copy of *pmoC* is present in *M. capsulatus* (Bath) (Stolyar *et al.*, 1999). Notably, the sequence of this third *pmoC* copy diverges from those of the other two copies. Gene disruption experiments using *M. capsulatus* (Bath) indicate that both *pmoCAB* gene copies are functional and that both are necessary for maximal pMMO activity. The third *pmoC* copy has been suggested to play an essential role in growth on methane, distinct from the functions of the other two *pmoC* genes (Stolyar *et al.*, 1999). The *pmo* genes are homologous to the genes encoding ammonia monooxygenase (AMO) (Holmes *et al.*, 1995), which also are present in two full copies as well as an extra copy of the gene analogous to *pmoC* (Stein *et al.*, 2000). Additional homologs to the *pmo* genes have not been identified, although similar monooxygenases may be present in butane-utilizing bacteria. These organisms do not oxidize methane, however (Hamamura *et al.*, 1999; Hamamura & Arp, 2000).

The genes for sMMO comprise the *mmoXYBZDC* operon (Stainthorpe *et al.*, 1989, 1990). The *mmoX*, *mmoY*, and *mmoZ* genes encode the α, β, and γ subunits of MMOH, respectively. MMOR, which controls electron transfer from NADH to the active site of MMOH (Merkx *et al.*, 2001), is encoded by *mmoC*. MMOB, which is required for activity (Merkx *et al.*, 2001), is encoded by *mmoB*. Proteins analogous to MMOR and MMOB have not been identified for the pMMO system. The function of the *mmoD* (also known as *orfY*) gene product is unknown, but this protein may play a role in assembly of the MMOH diiron active site (Merkx & Lippard, 2002).

REGULATION OF MMO

In methanotrophs that express both sMMO and pMMO, sMMO is expressed at low copper concentrations in the medium (<0.8 μM). At higher copper levels (~4 μM), pMMO is expressed and extensive intracytoplasmic membranes develop (Stanley *et al.*, 1983; Prior & Dalton, 1985). The addition of copper to both *M. capsulatus* (Bath) (Nielsen *et al.*, 1996) and *M. trichosporium* OB3b (Nielsen *et al.*, 1997) leads to a decrease in sMMO mRNA, suggesting that transcription of sMMO genes is specifically repressed by copper. Conversely, pMMO mRNA appearance is correlated with the presence of copper ions (Nielsen *et al.*, 1997). These data suggest that copper-binding repressor or activator proteins are involved in the switch between sMMO and pMMO expression (Murrell *et al.*, 2000a, 2000b). Although such proteins have not yet been identified, new genes involved in sMMO regulation have been discovered recently. A *groEL*-like bacterial chaperonin gene (*mmoG),* a σ^N-dependent transcriptional activator gene (*mmoR)*, and a two-component sensor–regulator system (*mmoQ* and *mmoS)*, are located downstream (3′) of the *M. capsulatus* (Bath) sMMO operon. (Figure 2; Csáki *et al.*, 2003).

The *mmoR* and *mmoG* genes are also present in *M. trichosporium* OB3b, although they are positioned upstream (5′) of the sMMO operon (Figure 2; Stafford *et al.*, 2003). The function of MmoG is unknown, and neither

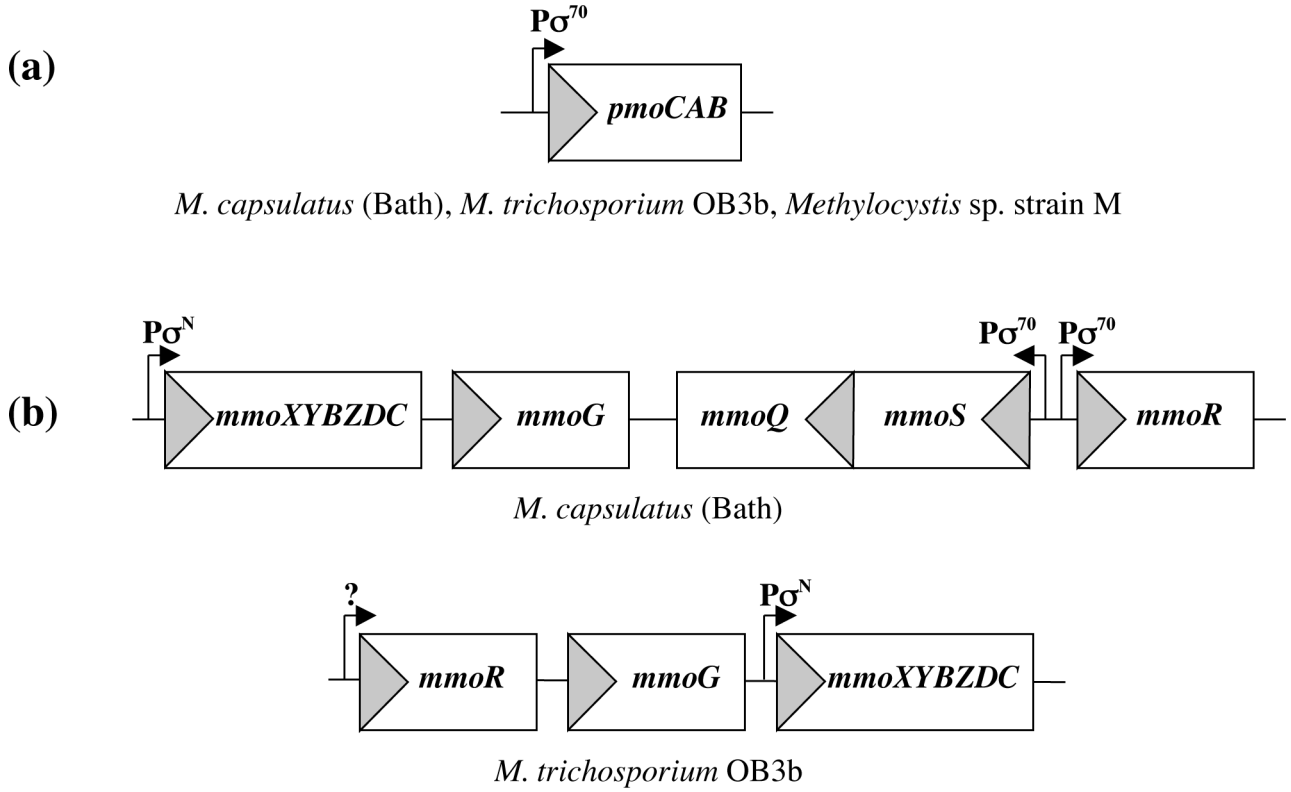

FIG. 2. Methane monooxygenase genes. (a) The *pmoCAB* operon encodes the pMMO proteins. Regulatory factors for pMMO have not been identified. (b) The *mmoXYBZDC* operon encodes the sMMO proteins. In *M. capsulatus* (Bath), the regulatory genes *mmoG*, *mmoQ*, *mmoS*, and *mmoR* are located downstream of the sMMO operon. In *M. trichosporium* OB3b, *mmoR* and *mmoG* are found upstream of the sMMO operon.

organism contains a companion *groES*-like gene adjacent to *mmoG*. MmoG may help assemble the sMMO complex or assist with MmoR folding (Figure 3). Alternatively, it could be a factor required for sMMO transcription (Csáki *et al.*, 2003; Stafford *et al.*, 2003). MmoR is proposed to activate transcription of the *mmoXYBZDC* operon via the σ^N-type promoter. The gene for the σ^N subunit of RNA polymerase, *rpoN*, has been identified in *M. trichosporium* OB3b (Stafford *et al.*, 2003). Although the promoter is copper-sensitive, no obvious copper-binding motifs are present in MmoR, leaving open the question of how MmoR responds to copper-limited conditions. Mutagenesis of *mmoG*, *mmoR*, and *rpoN* interferes with sMMO expression (Csáki *et al.*, 2003; Stafford *et al.*, 2003), supporting essential roles for these gene products in MMO regulation.

The copper-sensing mechanism might involve the sensor and regulator proteins encoded by *mmoS* and *mmoQ*, respectively (Figure 2b). These gene products exhibit ~50% sequence identity to two-component regulatory systems in which an environmental signal detected by the sensor protein is transmitted to the regulatory protein by transphosphorylation (Csáki *et al.*, 2003). Typical metal-binding motifs have not been identified in the sensing N-terminal region of MmoS or in MmoQ, however, and the proteins have not been characterized biochemically. Thus, in the current model of *M. capsulatus* (Bath) sMMO transcriptional regulation by copper (Figure 3), an unidentified protein or factor senses copper, transmits the signal to MmoS, which phosphorylates itself and then transfers the phosphoryl group to a receiver domain at the C-terminus of MmoQ. MmoQ is then proposed to regulate sMMO expression through an interaction with MmoR. It is also possible that the disappearance of sMMO upon copper addition is not solely due to transcriptional regulation. In one study, the addition of copper to *M. trichosporium* OB3b cultures expressing sMMO resulted in no detectable sMMO proteins by Western blot analysis within 24 h. Based on the rate of cell growth, repression of sMMO synthesis and dilution of the sMMO initially present should not have led to a complete disappearance of sMMO by this time point (Fitch *et al.*, 1993). Therefore, additional regulatory mechanisms might involve targeted degradation of sMMO in response to copper.

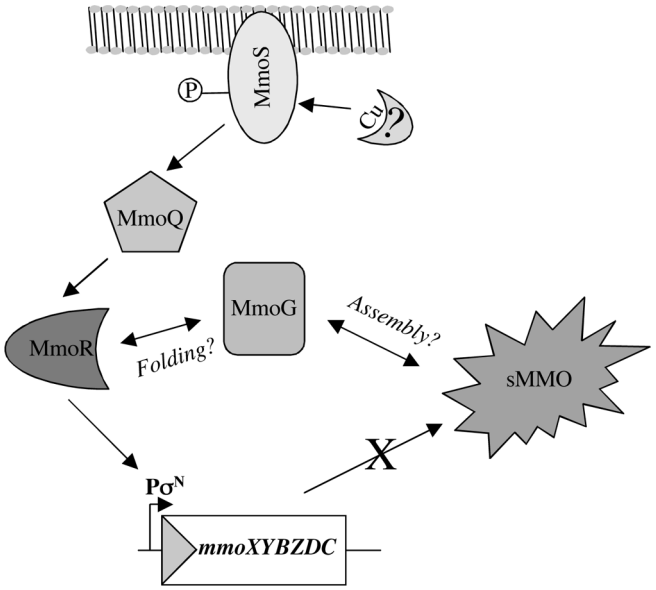

FIG. 3. Model for sMMO regulation. An unidentified sensor detects copper ions and transmits the signal to MmoS, which in turn signals MmoQ through phosphorylation. MmoQ then interacts with MmoR, which represses *mmoXYBZDC* transcription. MmoG may be involved in the assembly of sMMO or the folding of MmoR.

Regulatory proteins involved in pMMO expression have not yet been identified. Analysis of the *pmo* operons from *M. capsulatus* (Bath) (Stolyar *et al.*, 2001), *M. trichosporium* OB3b (Stafford *et al.*, 2003), and *Methylocystis* sp. strain M (Gilbert *et al.*, 2000) suggest that the three pMMO structural genes are transcribed from a single σ^{70} promotor upstream of *pmoC* (Figure 2a). Transcription does not appear to be controlled by σ^{N} or MmoR because *M. trichosporium* OB3b strains lacking the genes coding for these proteins still grow on methane at high copper concentrations, indicating that pMMO is expressed (Stafford *et al.*, 2003). Expression of the two *pmoCAB* copies appears to be regulated by copper levels. At 5 μM copper in the growth medium copy 2 transcripts are dominant, whereas at 50 μM copper, both copies are present at similar levels (Stolyar *et al.*, 2001). An understanding of the copper switch between sMMO and pMMO expression will require identification of the factors controlling pMMO expression. In addition, studies of regulation for methanotroph strains that only produce pMMO are currently lacking and may provide insight into more general aspects of pMMO expression. Detailed knowledge of MMO regulation would allow the engineering of strains that produce sMMO at high copper concentrations, exploiting the broader substrate specificity of sMMO (Burrows *et al.*, 1984) in the context of the high copper levels generally found in polluted environments (Phelps *et al.*, 1992).

COPPER UPTAKE

Despite the importance of copper in both the regulation and chemistry of pMMO, copper uptake systems have not been identified. Copper accumulation has been studied in *Methylomicrobium album* BG8 (also called *Methylomonas albus* BG8), a methanotroph that only expresses pMMO. The cells are rapidly saturated with 1–3 $\times$ 10^{-17} mol copper/cell, independent of copper levels in growth medium. This result is consistent with the existence of a specific copper transporter (Berson & Lidstrom, 1996). To search for such transporters, protein expression in *M. album* BG8 was investigated as a function of copper concentration in the growth medium (Berson & Lidstrom, 1997). Several proteins up- or downregulated by copper were identified by SDS-PAGE analysis, but only one copper-repressible 28.5 kDa polypeptide could be N-terminally sequenced. This protein, called CorA (Copper-repressible polypeptide A), has seven predicted transmembrane domains and bears some similarity to rabbit and human calcium-release channel proteins. No obvious copper-binding motifs were identified. Deletion of CorA led to poor cell growth, which could not be rescued by copper addition. Thus, CorA appears to be critical for *M. album* BG8 growth, but a definitive role in copper transport has not been established.

Additional evidence for copper transport systems in methanotrophs derives from studies of *M. trichosprium* OB3b mutants that do not express active pMMO or produce extensive intracellular membranes, but constitutively express active sMMO even in the presence of 5–12 μM copper (Phelps *et al.*, 1992). Although copper inhibits wildtype sMMO activity *in vitro* (Green *et al.*, 1985; Jahng & Wood, 1996), the soluble fractions of cell lysates from the mutants retain 77 $\pm$ 15% sMMO activity even in the presence of 15 μM copper. Some of the mutants exhibit 40 kDa and 24 kDa proteins in the particulate fraction, possibly corresponding to pMMO subunits, but these polypeptides are not copper inducible and no pMMO activity is observed (Phelps *et al.*, 1992). The overall speciation of copper also differs for the mutants. The mutants contained markedly less cell-associated copper, evenly distributed between the particulate and soluble fractions. By contrast, most of the copper associated with wildtype cells is found in the membrane fraction. This observation can be explained by the lack of active pMMO and the absence of extensive intracytoplasmic membranes in the mutants. Finally, the extracellular media from the mutants contained higher concentrations of solubilized copper. Although these phenotypes have not yet been linked to any specific genes, the data are consistent with several hypotheses. One possibility is that the mutations affect a general, copper-dependent regulatory system that controls copper uptake, differential MMO expression, and

production of intracytoplasmic membranes. Alternatively, *M. trichosporium* OB3b might excrete a copper complexing agent to recruit copper, analogous to an iron siderophore. In the mutant strains, this agent is either overproduced or cannot be internalized properly (Fitch *et al.*, 1993).

Candidates for this putative siderophore-like agent have been detected in *M. trichosporium* OB3b (DiSpirito *et al.*, 1998; Téllez *et al.*, 1998) and *M. capsulatus* (Bath) (Zahn & DiSpirito, 1996). These molecules, called copper-binding compounds (CBCs) or copper-binding ligands (CBLs), are abundant in spent media from cells grown at low copper concentrations. Upon copper supplementation, the CBC concentration in the media decreases, and the majority of the CBCs are associated with the membranes, consistent with a role in copper assimilation. Addition of copper does not affect the CBC concentration in the spent media of the *M. trichosporium* OB3b mutant strains that only express sMMO, suggesting that these mutants cannot import copper bound to these compounds (DiSpirito *et al.*, 1998; Téllez *et al.*, 1998). In addition to or instead of a copper import function, DiSpirito and coworkers suggest that the CBCs could play a direct role in methane oxidation or in protection against oxygen radicals (Zahn & DiSpirito, 1996; DiSpirito *et al.*, 1998; Choi *et al.*, 2003). In support of the latter notion, superoxide dismutase-like activity has been observed for the copper-loaded CBCs (Choi *et al.*, 2003).

The CBCs have been partially characterized. These compounds range in molecular mass from 382 to 1216 Da (DiSpirito *et al.*, 1998; Téllez *et al.*, 1998) and bind one copper ion (DiSpirito *et al.*, 1998) with high affinity (Téllez *et al.*, 1998). Although the complete chemical composition has not been reported, potential coordinating residues identified in the CBCs are methionine and tyrosine, and modified amino acids or other functional groups are suggested to be present (DiSpirito *et al.*, 1998). In the absence of copper, optical features at 204, 275, 333, and 356 nm are observed, with shoulders at 222 and 400 nm. Copper addition causes an increase in absorption in the 200–290 nm range. The copper-loaded CBCs have electron paramagnetic resonance (EPR) spectroscopic parameters between those for type 1 and type 2 copper, and in some compounds, two spectrally distinct centers appear to be present (DiSpirito *et al.*, 1998).

BIOCHEMISTRY OF pMMO

Isolation and Purification

Large quantities of pMMO are found in the intracytoplasmic membranes of methanotrophs cultivated in the presence of copper (Prior & Dalton, 1985; Brantner *et al.*, 2002). Nevertheless, difficulties in obtaining enzymatically active, pure pMMO have led to conflicting results of in vitro biochemical characterization. Therefore, a detailed discussion of isolation and purification procedures is warranted (Table 1). Although methanotrophs are typically grown in nitrate mineral salts media, the supplemental copper and iron concentrations used by different laboratories vary considerably and could affect subsequent biochemical analysis of membrane-bound and purified pMMO samples. Two recent studies systematically investigated the effects of copper levels in the growth medium on *M. capsulatus* (Bath) pMMO yield and activity (Choi *et al.*, 2003; Yu *et al.*, 2003a). DiSpirito and coworkers determined that cells grown with 60 μM $CuSO_4$ exhibit maximal membrane development, pMMO concentration per cell, and cell-free activity. In their optimized fermentation scheme, copper and media addition rates to a chemostat are adjusted to maintain a constant optical density and copper level (Choi *et al.*, 2003). By contrast, Chan and coworkers observe the highest pMMO expression and activity with cells grown in the presence of 30–35 μM copper and 18 μM iron. In their protocol, a hollow-fiber membrane bioreactor is used to filter off used media and maintain a constant copper concentration (Yu *et al.*, 2003a).

The least variable aspect of the reported purification procedures is isolation of the pMMO-containing membrane fraction. Cells are first lysed by sonication (Tonge *et al.*, 1977; Takeguchi *et al.*, 1998a, 1998b; Miyaji *et al.*, 2002; Xin *et al.*, 2002; Lieberman *et al.*, 2003), French press (Smith & Dalton, 1989; Nguyen *et al.*, 1994, 1996, 1998; Zahn & DiSpirito, 1996; Choi *et al.*, 2003; Yu *et al.*, 2003a) or cell disruptor (Basu *et al.*, 2003). After pelleting cell debris, the membrane fraction is isolated by ultracentrifugation. The membranes are generally washed 1–3 times to remove adventitious or loosely bound soluble proteins. Neutral pH buffers are used for these procedures, some containing reducing agents and catalase to improve enzyme stability (Nguyen *et al.*, 1998; Yu *et al.*, 2003a) and others containing 0.25–1.0 M NaCl or KCl (Nguyen *et al.*, 1994, 1996; Zahn & DiSpirito, 1996; Basu *et al.*, 2003; Choi *et al.*, 2003; Lieberman *et al.*, 2003).

All researchers isolating pMMO use the same activity assay, the epoxidation of propylene to propylene oxide (Colby *et al.*, 1977). In a typical assay, the pMMO-containing sample is mixed with a reductant and placed in a septum-sealed vial. A defined volume of air is then evacuated, replaced with propylene gas, and the vial is incubated at a set temperature for several minutes. The production of propylene oxide as a function of time is analyzed by gas chromatography. The most commonly used reductants are NADH and duroquinol (Shiemke *et al.*, 1995), with duroquinol shown to be particularly effective for measuring activity of detergent-solubilized pMMO (vide infra). In cells grown with 0–20 μM copper, addition of $CuSO_4$ or $CuCl_2$ prior to lysis or to membrane fractions has been shown to increase pMMO activity (Prior & Dalton, 1985;

TABLE 1
Purification protocols for pMMO

Organism laboratory	Lysis method	Crude membrane activity in nmol/mg·min (reductant)	Assay conditions	Detergent solubilization: Detergent	Detergent solubilization: mg detergent/mg protein	Chromatography column(s)	Purified pMMO activity in nmol/mg·min (reductant)	References
M. capsulatus (Bath)								
Chan	French press	12.5 (NADH)	45°C	DDM	2	L-Lysine agarose	5.1 (NADH)	Nguyen *et al.*, 1998
	French press	9.66 (NADH)	45°C	DDM	2	DEAE Sepharose FF	2.6 (NADH)	Nguyen *et al.*, 1998
	French press	88.9 (NADH)	45°C, 100 μM $CuSO_4$	DDM	1	Sephacryl S300 HR	21.5 (NADH), 15.6 (DQ)	Yu *et al.*, 2003a
Dalton	Cell disrupter	75–200 (NADH), 19 (DQ)	45°C, 50 μM $CuSO_4$	DDM	1.5	Superdex 200, DEAE cellulose	27.5 (DQ)	Basu *et al.*, 2003
DiSpirito	French press	10.4 (DQ)	37°C	DDM	1.4	DEAE cellulose, Phenyl Sepharose CL4B	11.08 (DQ)	Zahn and DiSpirito, 1996
	French press	44–118 (NADH), 40–86 (DQ)	42°C, 0.2–0.6 mol Cu/mol $\alpha\beta\gamma$ pMMO	DDM	1.2	DEAE Sepharose FF	64–126 (DQ)	Choi *et al.*, 2003
Rosenzweig	Sonication	21 (NADH), 16 (DQ)	42°C	DDM	2.5	Source 30Q, Sephacryl S200	17.7 (DQ)	Lieberman *et al.*, 2003
M. trichosporium OB3b								
Okura	Sonication	2.27 (DQ)	30°C	DDM	0.92	DEAE Sepharose FF Phenyl Sepharose 6FF	0.469 (DQ)	Takeguchi *et al.*, 1998b
	Sonication	3.8 (DQ)	30°C	Brij-58	n/a	Poros 20 HQ	3.4 (DQ)	Miyaji *et al.*, 2002

Nguyen *et al.*, 1994; Semrau *et al.*, 1995b; Xin *et al.*, 2002; Basu *et al.*, 2003). No effect is observed for cells grown with 50 μM copper, however (Lieberman *et al.*, 2003).

Purification of membrane-bound enzymes is difficult because removal from the hydrophobic environment of the lipid bilayer can cause loss of activity, either by altering the native protein conformation or by disrupting critical interactions with other proteins and lipids. Nondenaturing detergents are commonly used for solubilization, but the identity and concentration of the detergent are critical (Kühlbrandt, 1988; Michel, 1991). Dalton and coworkers were the first to successfully solubilize *M. capsulatus* (Bath) pMMO using the nonionic detergent dodecyl-β-D-maltoside (DDM) (Smith & Dalton, 1989). After solubilization and detergent removal, they were able to restore some enzyme activity by the addition of lecithin. Further purification attempts resulted in complete loss of enzyme activity, however. Since these initial efforts, seven different purification schemes for *M. capsulatus* (Bath) pMMO and two for *M. trichrosporium* OB3b pMMO have been reported (Table 1). In all of these protocols, DDM is used as the detergent (Zahn & DiSpirito, 1996; Nguyen *et al.*, 1998; Takeguchi *et al.*, 1998b; Basu *et al.*, 2003; Choi *et al.*, 2003; Lieberman *et al.*, 2003; Yu *et al.*, 2003a), with the exception of one procedure in which DDM is used for solubilization followed by Brij-58 for purification (Miyaji *et al.*, 2002). One additional purification procedure has been described for *M. trichosporium* IMV 3011 pMMO, but the report does not mention detergents and lacks analysis of the purified product by SDS-PAGE (Xin *et al.*, 2002). For solubilization, 1–2.5 mg DDM per mg of membrane pMMO have been used (Table 1), comparable to the optimal values of 1–1.25 mg per mg protein determined recently (Choi *et al.*, 2003; Yu *et al.*, 2003a). The DDM concentration in the purification buffers varies considerably, from 0.01 to 1%. Most protocols have been conducted aerobically, but in some cases, the use of degassed buffers (Yu *et al.*, 2003a) or an anaerobic chamber is reported (Zahn & DiSpirito, 1996; Choi *et al.*, 2003). Key details of the various methods are summarized below.

Two protocols yielding purified active *M. capsulatus* (Bath) pMMO have been developed by DiSpirito and coworkers (Zahn & DiSpirito, 1996; Choi *et al.*, 2003). The original protocol involves loading DDM-solubilized, membrane-bound pMMO onto a DEAE cellulose column apparently equilibrated without DDM (Zahn & DiSpirito, 1996). Fractions containing pMMO do not bind to the column, and are applied to a Phenyl Sepharose CL4B column and eluted with a high concentration of DDM. The purified pMMO comprises three subunits of molecular masses of 47, 27, and 25 kDa, and only minor contaminants are apparent by SDS-PAGE analysis. The identities of the three pMMO polypeptides were verified by N-terminal sequencing. The specific activity after purification was 7.8 $\pm$ 4.2 nmol/min·mg using duroquinol as the reductant. More recently, a modified purification protocol yielding highly active pMMO has been reported (Choi *et al.*, 2003). Starting with cells cultured using optimized fermentation procedures, pMMO is solubilized and loaded onto a DEAE cellulose column. In this protocol, the pMMO fraction adheres to the column until coelution with NADH dehydrogenase using a KCl gradient. Separation of pMMO and NADH dehydrogenase is accomplished using a DEAE Sepharose column. The specific activity of the NADH dehydrogenase-pMMO complex is 147 $\pm$ 43 nmol/min·mg and that of purified pMMO is 134 $\pm$ 36 nmol/min·mg.

Three purification protocols for *M. capsulatus* (Bath) pMMO have been reported by Chan and coworkers. In the first method, pMMO is purified to ~90% homogeneity and a specific activity of 5.1 nmol/mg·min using an L-lysine agarose column equilibrated with a buffer containing ascorbate and $CuSO_4$ (Nguyen *et al.*, 1998). To measure activity, the purified sample is reconstituted with lipids isolated from membrane suspensions. In the second method, pMMO is purified on DEAE Sepharose also equilibrated with a buffer containing ascorbate, $CuSO_4$, and sometimes dithionite (Nguyen *et al.*, 1998). Purified pMMO is eluted with a NaCl or NH_4Cl gradient and exhibits three prominent bands by SDS-PAGE corresponding to molecular masses of 45, 26, and 23 kDa and identified by N-terminal sequencing to be the pMMO subunits. A 35 kDa polypeptide, often present in these preparations, was determined to be a proteolytic fragment of the 45 kDa α subunit. The specific activity of lipid-reconstituted purified pMMO obtained by this procedure is 2.6 nmol/mg·min. For both procedures, the activity was found to vary from preparation to preparation. The third purification scheme uses cells grown under optimized conditions in the hollow-fiber membrane bioreactor (Yu *et al.*, 2003a). In this case, purified material is obtained by chromatography on Sephacryl S300 equilibrated with a buffer containing imidazole and ascorbate. Three polypeptides with molecular masses 45, 27, and 23 kDa were identified by trypsin digestion and peptide mass fingerprinting by MALDI-TOF mass spectrometry. The 35 kDa proteolytic fragment of the α subunit was observed in the membrane fractions, but not in the purified sample. Specific activities of 21.5 nmol/mg·min with NADH as the reductant, and 15.6 nmol/mg·min with duroquinol as the reductant were obtained. This report is the only one in which purified pMMO activity is higher using NADH than duroquinol.

Dalton and coworkers have isolated a pMMO complex composed of a hydroxylase (pMMOH) containing the pMMO α, β, and γ subunits and a putative reductase (pMMOR) comprising a 63 and an 8 kDa protein (Basu

et al., 2003). Purification of this complex on Superdex S200 gives a preparation with a specific activity of 27.5 nmol/mg·min. The pMMO complex can be further separated into pMMOH and pMMOR using DEAE cellulose. Neither the purified pMMOH nor the purified pMMO exhibits any activity on its own, and recombining the separated components yields a specific activity of 2.6 nmol/mg·min. The sizes of the pMMOR subunits and the N-terminal sequence of the 63 kDa component suggest that this complex might be a NAD-linked formaldehyde dehydrogenase (Tate & Dalton, 1999) or a methanol dehydrogenase. The mechanism by which this component mediates pMMO activity remains unclear.

Finally, we have purified *M. capsulatus* (Bath) pMMO in our laboratory using a Source 30Q column followed by a Sephacryl S200 column (Lieberman *et al.*, 2003). The purified pMMO consists of three subunits with molecular masses 47, 24, and 22 kDa. The 35 kDa proteolytic fragment observed by Chan and coworkers (Nguyen *et al.*, 1998) is not observed under any conditions. The 63 kDa putative formaldehyde or methanol dehydrogenase (Basu *et al.*, 2003) is typically removed prior to purification by washing the membranes. A specific activity of 17.7 nmol/mg·min was obtained, but variability in enzyme activity is observed, similar to that reported for other protocols (Nguyen *et al.*, 1998). Activity could not be recovered in inactive samples by a number of methods, including removal of bound detergent by dialysis or supplementing the assay mixture with reducing agents, $CuSO_4$, or CuCl. No activity was measured after lipid reconstitution, as described by Chan and coworkers (Nguyen *et al.*, 1998). Nevertheless, a circular dichroism (CD) spectrum of purified pMMO shows secondary structure, indicating that the purified protein is folded.

M. trichosporium OB3b pMMO has been purified in two ways by Okura and coworkers (Takeguchi *et al.*, 1998b; Miyaji *et al.*, 2002). The first protocol is similar to that of DiSpirito and coworkers (Zahn & DiSpirito, 1996), with a few modifications. Instead of DEAE cellulose, DEAE Sepharose is used. Like *M. capsulatus* (Bath) pMMO, the *M. trichosporium* OB3b enzyme does not bind to the column, and the flowthrough fraction is applied to a Phenyl Sepharose column. The purified pMMO is eluted with a DDM gradient and yields a specific activity of 0.469 nmol/mg·min. SDS-PAGE analysis reveals two bands, corresponding to molecular masses of 41 and 25 kDa, as well as trace amounts of a 26 kDa polypeptide. The identity of these subunits was not confirmed by N-terminal sequencing. In the second protocol, pMMO is first solubilized with an unspecified quantity of DDM (Miyaji *et al.*, 2002). The DDM is removed with BioBeads, and Brij-58 is added for purification using a POROS 20 HQ column. Neither the concentration of Brij-58 nor the buffer(s) used for purification is mentioned. Three polypeptides of molecular masses 43, 27, and 24 kDa are detected by SDS-PAGE, and a specific activity of 3.4 nmol/min·mg is reported.

Polypeptide Arrangement and Active Site Location

The organization of the three pMMO polypeptides, the subunit stoichiometry, and the total molecular mass of the holoenyzme have not been established unambiguously. Originally, it was assumed that the pMMO polypeptides from *M. capsulatus* (Bath) were present in a 1:1:1 ratio, corresponding to an $\alpha\beta\gamma$ heterotrimer of ~100 kDa. Chan and coworkers have determined a molecular mass for purified pMMO of 220 kDa by gel filtration chromatography. These data were interpreted as a 100 kDa $\alpha\beta\gamma$ monomer and 240 DDM detergent molecules (Yu *et al.*, 2003a). The molecular mass of a multisubunit membrane protein is difficult to measure by analytical gel filtration, however (Hjelmeland & Chrambach, 1984; le Maire *et al.*, 2000). These data are also consistent with a molecular mass of ~180 kDa and the presence of ~80 DDM molecules. In support of this interpretation, we have obtained a molecular mass of ~200 kDa for purified pMMO using two gel electrophoresis techniques (Lieberman *et al.*, 2003), PFO (perfluorooctanoic acid)-PAGE (Ramjeesingh *et al.*, 1999) and BN (blue native)-PAGE (Schägger & von Jagow, 1991). These techniques are similar to SDS-PAGE except that the dodecylsulfate anion is replaced with a nondenaturing alternative. In the PFO-PAGE experiment, the ~200 kDa band is accompanied by a second ~47 kDa band, suggesting that PFO is partially denaturing under the experimental conditions. The BN-PAGE experiment reveals an additional ~440 kDa band that could represent a tetramer. A 100 kDa band was not observed in either experiment. The presence of the ~200 kDa band in both experiments suggests that purified pMMO has an $\alpha_2\beta_2\gamma_2$ polypeptide structure, although other arrangements cannot be excluded.

The molecular mass of *M. trichosporium* OB3b pMMO has been determined to be 326 kDa by analytical gel filtration (Takeguchi *et al.*, 1998b; Takeguchi & Okura, 2000). The 326 kDa complex comprises two subunits with molecular masses of 25 and 41 kDa. Since this preparation also contains trace amounts of a 26 kDa polypeptide (vide supra), the subunit composition remains unclear. Another protocol reported by the same group using the detergent Brij-58 results in a predicted molecular mass of 94 kDa (Miyaji *et al.*, 2002). Finally, pMMO from *M. trichosporium* IMV 3011 is reported to have a molecular mass of 96 kDa by gel filtration (Xin *et al.*, 2002).

The location of the active site within the pMMO complex is not known, but radiolabeling studies with the suicide substrate acetylene have provided some insight.

TABLE 2
Metal ion analysis of purified pMMO

Organism	Laboratory	Mol Cu per 200 kDa	Mol Fe per 200 kDa	References
Methylococcus capsulatus (Bath)				
	Chan	24.8	~0	Nguyen *et al.*, 1998
		30	~0	Nguyen *et al.*, 1998
		27.2	~0	Yu *et al.*, 2003a
	Dalton	4	2	Basu *et al.*, 2003
	DiSpirito	29	5	Zahn and DiSpirito, 1996
		16–20	4	Choi *et al.*, 2003
	Rosenzweig	4–6	1–2	Lieberman *et al.*, 2003
Methylosinus trichosporium OB3b				
	Okura	25.6	1.8	Takeguchi *et al.*, 1998b
		4	0	Miyaji *et al.*, 2002

Experiments on *M. capsulatus* (Bath) pMMO suggest that the active site is housed on the ~24 kDa β subunit (Cook & Shiemke, 1996; Zahn & DiSpirito, 1996) and may also involve the ~47 kDa α subunit (Zahn & DiSpirito, 1996). A ~28 kDa polypeptide is labeled in similar experiments with AMO from *Nitrosomonas europaea* (Hyman & Wood, 1985), supporting the proposal that this subunit contains the active site. Moreover, a ~30 kDa polypeptide is labeled with acetylene in cultures of butane-oxidizing bacteria suspected to contain a butane monooxygenase with some similarity to pMMO and AMO (Hamamura *et al.*, 1999; Hamamura & Arp, 2000).

THE pMMO ACTIVE SITE

Metal Ion Content

Although pMMO is generally believed to be a metalloenzyme, the metal composition and stoichiometry are controversial. All reported metal analyses indicate the presence of copper. The discussion of metal ion analysis here is reported relative to a 200 kDa complex (Table 2), unless stated otherwise. For membrane-bound pMMO from *M. capsulatus* (Bath), values of 8–118 copper ions have been reported (Nguyen *et al.*, 1994, 1996; Semrau *et al.*, 1995b; Zahn & DiSpirito, 1996; Lieberman *et al.*, 2003; Yu *et al.*, 2003a). The addition of excess EDTA reduces the specific activity by ~50% (Basu *et al.*, 2003; Lieberman *et al.*, 2003), suggesting that the copper ions are necessary for activity, but may differ in accessibility. Upon purification, values of 24–30 copper ions (Zahn & DiSpirito, 1996; Nguyen *et al.*, 1998; Yu *et al.*, 2003a) and 4–6 copper ions per 200 kDa have been obtained (Basu *et al.*, 2003; Lieberman *et al.*, 2003). For one preparation, 16–20 copper ions were measured, but 4 were attributed to pMMO and the remaining 12–16 to the CBCs (Figure 4; Choi *et al.*, 2003). The decreased copper content in some purified samples may be due to more extensive washing of both the harvested cells and membrane fractions, removing large amounts of adventitiously bound copper or associated CBCs. In addition, different methods for measuring the protein concentration could account for discrepancies. In several studies, the protein concentration was determined using the detergent-compatible version of the Lowry assay (Basu *et al.*, 2003; Lieberman *et al.*, 2003) or the extinction coefficient obtained from amino acid hydrolysis (Lieberman *et al.*, 2003). These methods may be more accurate for detergent-containing samples than the Bradford (Bradford, 1976) or other conventional protein assays.

A range of copper contents has also been reported for *M. trichosporium* OB3b samples. For the two-subunit 326 kDa complex, 18 and 8 copper ions were measured for membrane-bound and purified samples, respectively (Takeguchi *et al.*, 1998b, 1999b; Takeguchi & Okura, 2000). These numbers were later revised to 2 copper ions per purified 94 kDa complex (Miyaji *et al.*, 2002). A

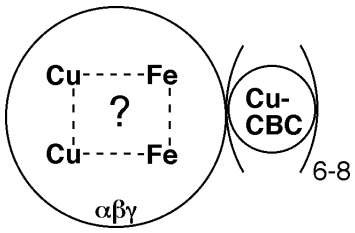

FIG. 4. Active-site model proposed by DiSpirito and coworkers. Each $\alpha\beta\gamma$ pMMO contains two copper and two iron ions, and the remainder of the copper ions are associated with the CBCs.

stoichiometry of 17 copper ions per 96 kDa has been reported for the putative purified *M. trichosporium* IMV 3011 pMMO (Xin *et al.*, 2002). Finally, pMMO-containing membrane fractions from *M. album* BG8 are estimated to contain ~4 copper ions per 99 kDa enzyme (Yuan *et al.*, 1998). If all the reported measurements for purified pMMO are normalized to a 200 kDa complex, the copper content ranges from 4 to 34 copper ions. Additional and more thorough studies are clearly required to establish the true copper content.

A second major question regarding the pMMO metal centers is the possible presence of iron. In some preparations of purified pMMO from *M. capsulatus* (Bath) (Zahn & DiSpirito, 1996; Basu *et al.*, 2003; Choi *et al.*, 2003; Lieberman *et al.*, 2003) and *M. trichosporium* OB3b (Takeguchi *et al.*, 1999b; Takeguchi & Okura, 2000), 1–4 iron ions per 200 kDa have been detected. The addition of Fe(III) to EDTA-treated *M. trichosporium* OB3b membranes (Takeguchi & Okura, 2000) or to *M. capsulatus* (Bath) preparations isolated from cells grown at low iron concentrations (Zahn & DiSpirito, 1996) increased pMMO activity, suggesting that Fe(III) is a necessary cofactor. DiSpirito and coworkers have proposed that the catalytic center involves both copper and iron, with the majority of the copper ions associated with pMMO by complexation with the CBCs (Figure 4). Other purified samples are reportedly iron free (Nguyen *et al.*, 1998; Miyaji *et al.*, 2002; Xin *et al.*, 2002; Yu *et al.*, 2003a), although iron is observed in membrane-bound samples (Nguyen *et al.*, 1996; Yu *et al.*, 2003a). Chan and coworkers have suggested that >2 μM iron in the growth medium leads to contamination by sMMO and other iron-containing proteins (Nguyen *et al.*, 1996, 1998). Preparations of pMMO obtained from cells grown with 80 μM FeEDTA do not contain sMMO, however. Other contaminants, such as cytochromes, could account for the presence of iron, but a functional role is also possible (Lieberman *et al.*, 2003).

Spectroscopic Studies

Numerous attempts to characterize the pMMO copper center(s) using electron paramagnetic resonance (EPR) spectroscopy have been reported. Chan and coworkers observe a typical type 2 Cu(II) signal and an isotropic signal at $g = 2.06$. The hyperfine splitting pattern of the isotropic signal is interpreted as trinuclear mixed-valence copper center consisting of one Cu(II) ion and two Cu(I) ions (Nguyen *et al.*, 1994, 1996). Based on these data and a copper stoichiometry for membrane preparations of 15–20 copper ions per 100 kDa, they propose that the pMMO metal centers are arranged in 5–7 trinuclear clusters (Figure 5). These clusters are further postulated to fall into two functional classes, designated the catalytic (C) and electron transfer (E) clusters. The C clusters are suggested to bind dioxygen and oxidize methane, whereas the E clusters provide reducing equivalents (Nguyen *et al.*, 1996). A similar EPR signal has been observed for purified pMMO from *M. trichosporium* OB3b and attributed to a trinuclear copper cluster (Takeguchi *et al.*, 1998a, 1998b; Takeguchi & Okura, 2000). However, an improved purification protocol resulted in the disappearance of this signal (Miyaji *et al.*, 2002).

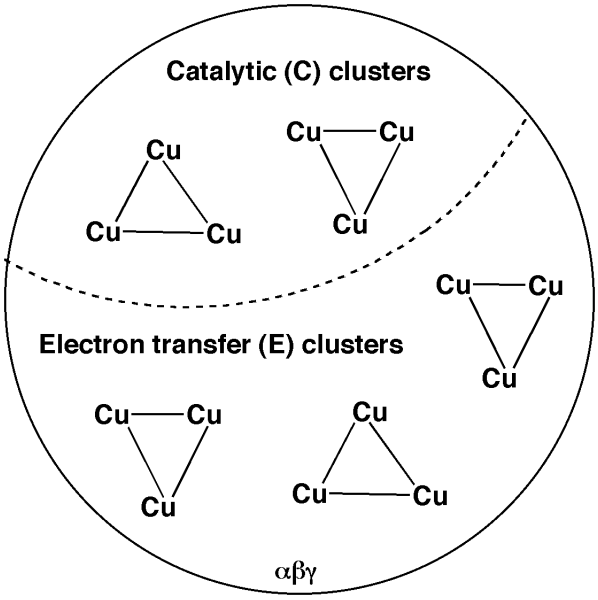

FIG. 5. Active-site model proposed by Chan and coworkers. The copper ions are arranged in 5–7 trinuclear clusters per $\alpha\beta\gamma$ pMMO and are postulated to fall into two functional classes, the catalytic (C) and the electron transfer (E) clusters.

In contrast to the findings of Chan and coworkers, other researchers only observe the typical type 2 Cu(II) EPR signal for purified *M. capsulatus* (Bath) pMMO (Zahn & DiSpirito, 1996; Basu *et al.*, 2003; Choi *et al.*, 2003; Lieberman *et al.*, 2003) as well as for membrane-bound pMMO and whole cell suspensions from *M. capsulatus* (Bath), *M. album* BG8 pMMO (Yuan *et al.*, 1997, 1999; Lemos *et al.*, 2000), and *M. capsulatus* strain M (Katterle *et al.*, 2002). Antholine and coworkers suggest that the isotropic signal attributed to a trinuclear copper cluster instead results from a radical and a mononuclear type 2 Cu(II) center (Yuan *et al.*, 1997). Electron spin echo modulation spectroscopic (ESEEM) data for *M. capsulatus* (Bath) and *M. album* BG8 whole cells indicate that the mononuclear type 2 Cu(II) center is coordinated by three or four histidine imidazoles (Lemos *et al.*, 2000). Similarly, ESEEM data for EDTA-treated *M. trichosporium* OB3b membranes indicates the presence of at least two imidazole ligands (Takeguchi *et al.*, 1999a). Histidine ligation is

also suggested by ESEEM data for *M. capsulatus* (Bath) membrane samples, but in this case it was attributed to the putative C clusters (Elliott *et al.*, 1998). Notably, addition of excess copper to partially purified EDTA-treated pMMO resulted in an artificial broad, isotropic signal similar to that attributed to a trinuclear cluster by Chan and coworkers (Basu *et al.*, 2003).

EPR quantitation of Cu(II) in different pMMO preparations has yielded varying results. For *M. album* BG8 (Yuan *et al.*, 1998; Lemos *et al.*, 2002) and *M. capsulatus* (Bath) (Nguyen *et al.*, 1996) membrane-bound samples, ~25% of the total copper is EPR detectable. All of the copper in purified pMMO from *M. trichosporium* OB3b is EPR active (Miyaji *et al.*, 2002), but samples of purified (Lieberman *et al.*, 2003) and partially purified (Basu *et al.*, 2003) *M. capsulatus* (Bath) pMMO contain 40–60% and <30% Cu(II), respectively. Reduction with dithionite consistently leads to a significant decrease in EPR signal intensity (Nguyen *et al.*, 1994, 1996; Yuan *et al.*, 1998; Lieberman *et al.*, 2003). By constrast, oxidation of pMMO has been challenging. Upon the addition of ferricyanide to *M. capsulatus* (Bath) membrane preparations, Chan and coworkers observe enhancement of the isotropic signal attributed to the trinuclear clusters (Nguyen *et al.*, 1996, 1998), and propose that all the clusters are oxidized (Nguyen *et al.*, 1998). However, Antholine and coworkers observe a similar signal when ferrocyanide is added to membrane fractions from other bacteria or to copper-loaded serum albumin. Their data therefore suggest that ferricyanide treatment results in formation of a nonspecific cupric ferrocyanide complex, rather than oxidation of a pMMO copper cluster (Yuan *et al.*, 1998). Unlike the membrane samples, ferricyanide addition to purified (Lieberman *et al.*, 2003) or partially purified pMMO (Basu *et al.*, 2003) does not affect the intensity of the Cu(II) EPR signal.

No information regarding specific histidine residues or other amino acid ligands to the copper ions is available, but sequence alignments suggest some possibilities. Only three histidines, His 38, His 40, and His 168, are strictly conserved in the β subunit (*pmoA*), which is proposed to house the active site (Figure 6; Cook & Shiemke, 1996; Zahn & DiSpirito, 1996). Five conserved histidines are present in the α subunit (*pmoB*), and four are present in the γ subunit (*pmoC*). These histidines are also conserved in the corresponding ammonia monooxygenase subunits. Even if the copper clusters are located in all three subunits, it is unclear how 12 histidine residues can accommodate the 5–7 trinuclear clusters proposed by Chan and coworkers (Nguyen *et al.*, 1996). Peptide amide nitrogens might also be involved in coordination. In terms of other possible copper ligands, there are seven conserved aspartic acid residues and four conserved glutamic acid residues in the β subunit. The α and γ subunits each contain eight strictly conserved carboxylate residues. There are no cysteines in the α subunit, and one each in the β and γ subunits of *M. capsulatus* (Bath) pMMO. These two cysteines are not conserved. Excluding the N-terminal methionines, there is one conserved methionine each in the α and β subunits and two conserved methionines in the γ subunit. There is no experimental evidence for sulfur ligation, however (Lieberman *et al.*, 2003).

Finally, the presence of iron in pMMO has been investigated by EPR. In most pMMO membrane preparations, a signal at $g = 4.3$, attributable to rhombic iron, is observed (Nguyen *et al.*, 1994; Zahn & DiSpirito, 1996; Takeguchi *et al.*, 1998b; Basu *et al.*, 2003; Lieberman *et al.*, 2003). DiSpirito and coworkers report that the addition of nitric oxide to purified pMMO samples yields an EPR signal characteristic of ferrous-NO complexes (Zahn & DiSpirito, 1996). Similar results were obtained using *M. trichosporium* OB3b membranes (Takeguchi & Okura, 2000), but they could not be reproduced for partially purified pMMO (Basu *et al.*, 2003). Surprisingly, reduction of partially purified pMMO with ascorbate and dithionite led to an increase in intensity of the $g = 4.3$ signal. Dalton and coworkers attribute this increase to Fe(III) interacting with another paramagnetic species in the as-isolated state, rendering it EPR silent. Interestingly, the saturation behavior for this signal is similar to that observed for the Fe(III)-tyrosinate center in transferrin (Basu *et al.*, 2003). No EPR signatures attributable to dinuclear, carboxylate-bridged, or iron-sulfur clusters have been observed.

The oxidation state and coordination environment of the pMMO copper ions have also been probed by X-ray absorption spectroscopy (XAS). The Cu X-ray absorption near edge structure (XANES) spectra of *M. capsulatus* (Bath) membrane-bound pMMO reveal a shoulder at ~8,984 eV (Nguyen *et al.*, 1996), attributable to a Cu(I) $1s \rightarrow 4p$ transition (Kau *et al.*, 1987). Quantitation of the edge spectra using dithionite-reduced and ferricyanide-oxidized samples for calibration indicates that $70 \pm 10\%$ of the total copper exists as Cu(I) (Nguyen *et al.*, 1996). In addition, we have analyzed purified *M. capsulatus* (Bath) pMMO by XAS (Lieberman *et al.*, 2003). The XANES spectra for purified and purified dithionite-reduced pMMO also exhibit ~8,984 eV absorption features, which are more pronounced than that observed for membrane-bound pMMO (Nguyen *et al.*, 1996). Therefore, some fraction of the copper in both samples is present in the Cu(I) oxidation state, consistent with EPR quantitation (Lieberman *et al.*, 2003). Since full oxidation of purified pMMO has not yet been achieved, the relative amounts of Cu(I) and Cu(II) cannot be determined from the XANES spectra of purified samples.

Two strong peaks are observed in the Fourier transform of the EXAFS data for purified *M. capsulatus* (Bath)

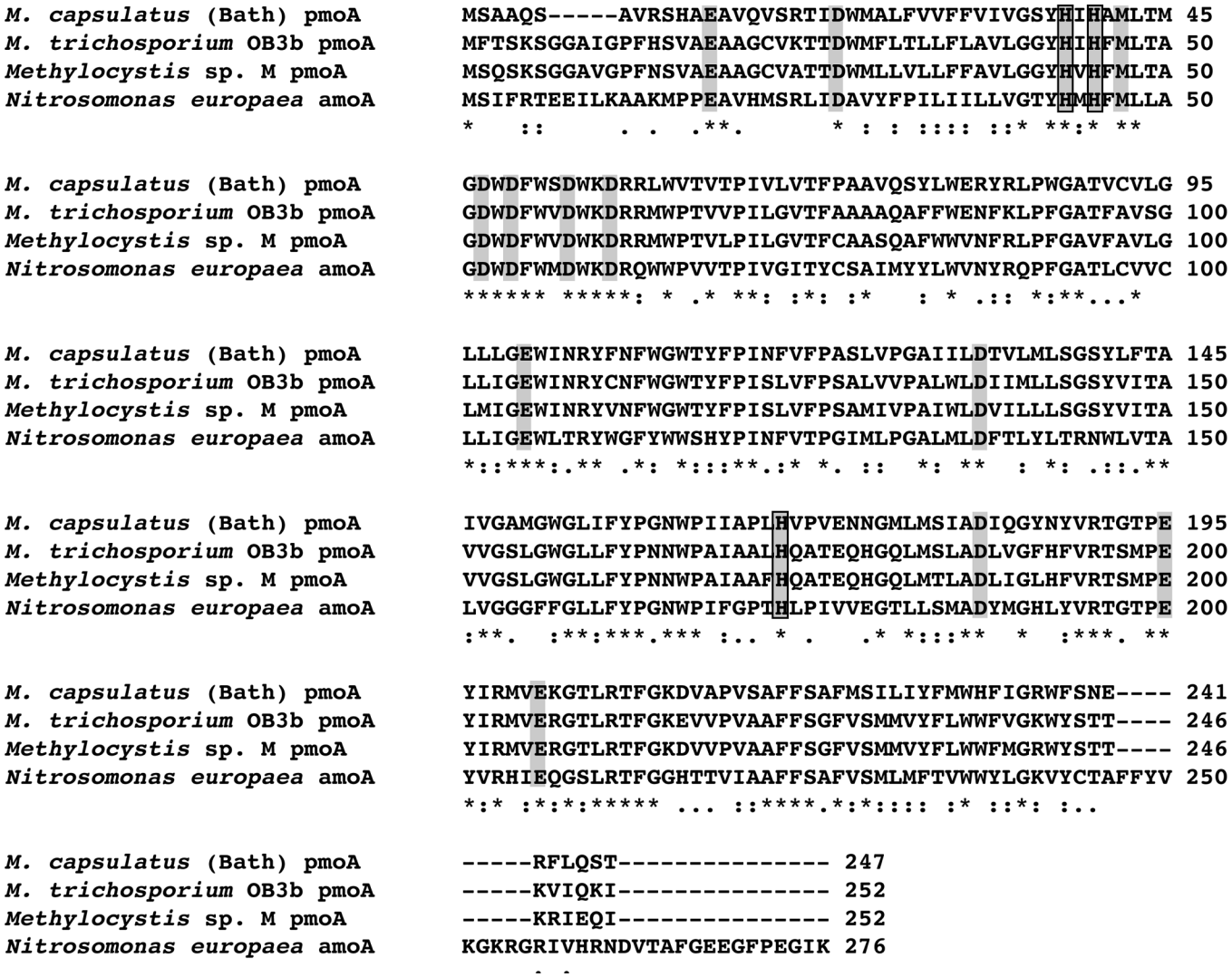

FIG. 6. Alignment of three pMMO β subunit (encoded by *pmoA*) sequences and the AMO β subunit sequence (encoded by *amoA*). The β subunit is proposed to house the active site. The three conserved histidine residues are highlighted gray and enclosed in black boxes. Other highly conserved potential liganding residues are highlighted gray. Completely conserved residues are denoted with asterisks, strongly conserved residues are denoted with double dots, and weakly conserved residues are denoted with single dots.

pMMO (Lieberman *et al.*, 2003). The first shell of backscatterers is best fit with oxygen/nitrogen (O/N) ligands at average bond lengths of 1.97 Å. The second peak corresponds to a second backscattering shell at ~2.6 Å. This feature was best fit by a Cu-Cu interaction at 2.57 Å. This short Cu-Cu distance is similar to those observed in the nitrous oxide reductase tetranuclear Cu_Z cluster (Brown *et al.*, 2000) and in the cytochrome *c* oxidase (Wilmanns *et al.*, 1995) and nitrous oxide reductase (Brown *et al.*, 2000) Cu_A sites. Optical and EPR spectroscopic features characteristic of Cu_A (Farrar *et al.*, 1996) or Cu_Z (Prudêncio *et al.*, 2000) are not observed for pMMO, however. The pMMO copper center may therefore represent a new type of biological copper cluster. Although it is likely that the interacting metal ion in pMMO is copper, further analysis is needed to determine whether another metal ion, such as iron, is involved. Nevertheless, our EXAFS data combined with information from EPR suggest that the active site may contain both mononuclear type 2 copper and a copper-containing cluster (Figure 7).

Catalytic Mechanism

MMOs catalyze the conversion of methane and dioxygen to methanol and water, with one atom of the dioxygen molecule being incorporated into methanol and the other

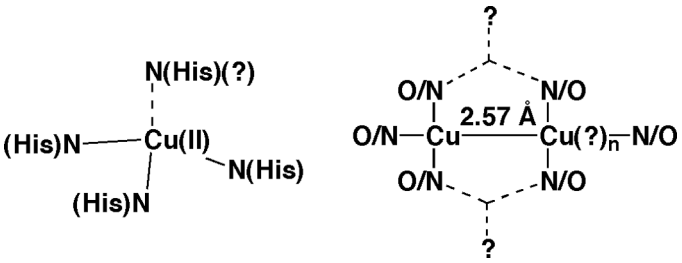

FIG. 7. Active-site model based on EPR and EXAFS data. The identity and number of the interacting metal ion(s) in the cluster has not yet been established.

into water. Three general mechanisms have been considered for this reaction: a radical mechanism, a carbocation intermediate mechanism, and a concerted oxenoid or oxene-insertion mechanism (Wilkinson *et al.*, 1996; Elliott *et al.*, 1997; Baik *et al.*, 2003). In the case of sMMO, extensive studies using purified enzyme and various mechanistic probes have provided some evidence for both radical and cationic intermediates. Studies with chiral alkanes have implied that the mechanism also has some concerted character, however (Priestley *et al.*, 1992; Valentine *et al.*, 1997). By contrast, there is no evidence for radical or cationic intermediates in the pMMO reaction analyzed using membrane preparations. Hydroxylation of both chiral ethane (Wilkinson *et al.*, 1996) and chiral butane (Yu *et al.*, 2003b) proceeds with full retention of configuration, consistent with a concerted mechanism and suggesting that mechanisms involving even very short-lived radical or cation intermediates are unlikely. Studies of hydrogen/deuterium kinetic isotope effects are also consistent with a concerted oxygen-insertion mechanism for pMMO (Wilkinson *et al.*, 1996; Yu *et al.*, 2003b). No carbon kinetic isotope effect has been observed for propane oxidation, suggesting that there is minimal structural change at the carbon center during transition state formation in the rate-limiting step (Huang *et al.*, 2002). Based on these data, Chan and coworkers propose direct insertion of an active "O" species across the C–H bond (Huang *et al.*, 2002). They further suggest that the oxene is delivered side-on to a bis-μ-dioxo-bridged dinuclear Cu(III) cluster (Yu *et al.*, 2003b).

The substrate specificity of pMMO has also been investigated. Whereas sMMO can oxidize a range of substrates besides methane, including saturated and unsaturated alkanes and halogenated, aromatic, and heterocyclic compounds (Baik *et al.*, 2003), pMMO can only oxidize substrates containing up to five carbons (Burrows *et al.*, 1984; Smith & Dalton, 1989; Nguyen *et al.*, 1996). Activity assays using a range of substrates and membrane preparations indicate some regio- and stereoselectivity (Elliott *et al.*, 1997). In particular, hydroxylation of propane, *n*-butane, and *n*-pentane favors the C-2 position. For *n*-butane and *n*-pentane, the (*R*)-alcohol is formed preferentially. Less selectivity was observed for alkene substrates. Overall, these studies suggest that the pMMO active site, although likely smaller than that in sMMO, can accommodate multiple substrate-binding modes (Elliott *et al.*, 1997). Since all the mechanistic studies of pMMO to date have been conducted using membrane preparations, further work on the purified enzyme is warranted. In addition, future mechanistic studies will benefit from a more detailed knowledge of the active site.

UNRESOLVED ISSUES

This review describes the current state of knowledge regarding the regulation, biochemistry, active site structure, and chemical mechanism of pMMO. Although important advances have been reported in recent years, the characterization of pMMO remains mired in controversy and unanswered questions. Key issues that need to be addressed include:

- What is the copper sensor controlling the switch between pMMO and sMMO expression? How is expression of pMMO regulated?
- How do methanotrophs acquire copper? What are the structure and function of the CBCs?
- How can pMMO activity be further improved in solubilized and purified preparations?
- Which pMMO subunit(s) house the active site(s)? How do substrates access the active site?
- What is the nature of the pMMO active site(s)? How many copper ions are present, how are they arranged, and what are the amino acid ligands? Are any exogenous ligands involved?
- What other metal ions, such as iron, are present in pMMO? How are they arranged and what is their function?
- What is the chemical mechanism of methane hydroxylation by pMMO?

Significant research efforts will be required to address these questions adequately. The answers will not only expand our understanding of biological metal centers and the chemistry of highly reactive species but will also impact bioremediation and the development of new industrial catalysts.

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