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Architecture and active site of particulate methane monooxygenase

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Abstract

Particulate methane monooxygenase (pMMO) is an integral membrane metalloenzyme that oxidizes methane to methanol in methanotrophic bacteria, organisms that live on methane gas as their sole carbon source. Understanding pMMO function has important implications for bioremediation applications and for the development of new, environmentally friendly catalysts for the direct conversion of methane to methanol. Crystal structures of pMMOs from three different methanotrophs reveal a trimeric architecture, consisting of three copies each of the pmoB, pmoA, and pmoC subunits. There are three distinct metal centers in each protomer of the trimer, mononuclear and dinuclear copper sites in the periplasmic regions of pmoB and a mononuclear site within the membrane that can be occupied by copper or zinc. Various models for the pMMO active site have been proposed within these structural constraints, including dicopper, tricopper, and diiron centers. Biochemical and spectroscopic data on pMMO and recombinant soluble fragments, denoted spmoB proteins, indicate that the active site involves copper and is located at the site of the dicopper center in the pmoB subunit. Initial spectroscopic evidence for O_2 binding at this site has been obtained. Despite these findings, questions remain about the active site identity and nuclearity and will be the focus of future studies.

Keywords

copper; methanotroph; membrane protein; dioxygen activation; ammonia monooxygenase; Cu-ZSM-5 zeolite; hemocyanin; tyrosinase

Introduction

Importance of biological methane oxidation

Methanotrophic bacteria oxidize methane to methanol in the first step of their metabolic pathway (Hanson & Hanson, 1996). Methane is a potent greenhouse gas, with a global warming potential more than 20 times that of carbon dioxide, and as the only biological methane sink, methanotrophs play a critical role in the global carbon cycle (Jiang et al., 2010). In addition, harnessing biological mechanisms of methane oxidation is becoming a pressing goal in the development of "green" catalysts. The notion of a "methanol economy" (Olah, 2005) could perhaps be realized if more efficient and environmentally benign catalytic processes existed for the direct and selective oxidation of methane to methanol.

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Current catalysts that activate the 104 kcal mol^{-1} C-H bond in methane require harsh conditions and produce waste (Arakawa et al., 2001). By contrast, methanotrophs perform this chemistry under ambient conditions, and thus their methane metabolizing enzymes may provide inspiration for the design of new catalysts.

Methanotrophs

Methanotrophs are divided into three phylogenetic groups on the basis of their carbon assimilation pathways, cell morphology, membrane arrangement, 16S RNA sequences, and other metabolic characteristics (Hanson & Hanson, 1996; Semrau et al., 2010). The type I methanotrophs, including the genera *Methylomicrobium* and *Methylobacter*, assimilate formaldehyde as a carbon source via the ribulose monophosphate pathway (RuMP) whereas the type II methanotrophs, including the genera *Methylocystis*, *Methylosinus*, and *Methylocella*, utilize the serine pathway. The type X methanotrophs, such as *Methylococcus,* use the RuMP, but also possess low levels of the serine pathway enzyme ribulose-bisphosphate carboxylase. Beyond the proteobacterial methanotrophs, filamentous methane oxidizers *Crenothrix polyspora* (Stoecker et al., 2006) and *Clonothrix fusca* (Vigliotta et al., 2007)) and acidophilic methanotrophs belonging to the Verrucomicrobia phylum (Op den Camp et al., 2009; Pol et al., 2007; Dunfield et al., 2007; Islam et al., 2008) have been discovered recently.

Methanotrophs are found in a wide variety of environments, and can oxidize a wide variety of other substrates, including halogenated hydrocarbons (Semrau et al., 2010), and thus represent a target for bioremediation applications. They grow at temperatures ranging from 4 °C to 72 °C and at extremes of pH and salt levels (Semrau et al., 2008; Semrau et al., 2010). Although most methanotrophs are obligate (only grow on one-carbon compounds), some strains are facultative and can grow on multicarbon compounds as well (Dedysh & Dunfield, 2011). The first methantroph genome to be sequenced was that of *Methylococcus capsulatus* (Bath) in 2004 (Ward et al., 2004). Since then, representative genomes of a wide variety of methanotrophs have been completed, including those of *Methylosinus trichosporium* OB3b (Stein et al., 2010), *Methylocystis* species strain Rockwell (Stein et al., 2011), *Methylocella silvestris* BL2 (Chen et al., 2010), *Methylomonas methanica* MC09 (Boden et al., 2011), *Methylobacter tundripaludum* SV96 (Svenning et al., 2011), *Methylomicrobium alcaliphilum* 20Z (Vuilleumier et al., 2012), and *Methylacidiphilum infernorum* (Hou et al., 2008). The combination of genomic data with the growing phylogenetic distribution of methanotrophs suggests that methanotrophs are more diverse in the environment than previously imagined (Jiang et al., 2010) and may produce unusual forms of metabolic enzymes, including methane monooxygenases (MMOs).

Methane monooxygenases

The oxidation of methane to methanol, the first step in the metabolic pathway of methanotrophs, is catalyzed by MMO enzymes. Methanol is further converted to formaldehyde by methanol dehydrogenase, and formaldehyde is then oxidized to formate and carbon dioxide by formaldehyde and formate dehydrogenases, respectively. Formaldehyde is also assimilated for biosynthesis of cell material composed of multicarbon compounds (Hanson & Hanson, 1996). There are two types of MMOs. The soluble form (sMMO) (Merkx et al., 2001) is expressed under conditions of copper limitation, and is only present in a few methanotroph strains. The membrane-bound form, referred to as particulate MMO (pMMO) (Lieberman & Rosenzweig, 2004; Balasubramanian & Rosenzweig, 2007; Hakemian & Rosenzweig, 2007), is expressed under copper-replete conditions and is present in all known methanotrophs with the exception of *Methylocella silvestris* BL2 (Theisen et al., 2005). In those organisms that possess both sMMO and pMMO, the switch in expression is copper-dependent (Ali & Murrell, 2009) (Choi et al., 2003; Prior & Dalton, 1985; Stanley

et al., 1983). Although there is some information on the genes and gene products involved (Csáki et al., 2003; Stafford et al., 2003; Ukaegbu et al., 2006; Ukaegbu & Rosenzweig, 2009), the specific mechanism of this switch has not been determined.

The two MMO systems are completely different in sequence, overall structure, substrate selectivity, and active site composition. sMMO is a multicomponent monooxygenase composed of a hydroxylase (MMOH), which houses the active site, a reductase (MMOR), which shuttles electrons from NADH to the active site of MMOH, and a regulatory protein (MMOB) that is required for activity (Merkx et al., 2001). MMOH consists of three polypeptides arranged in an $\alpha_2\beta_2\gamma_2$ dimer (Rosenzweig et al., 1993). Methane and O_2 bind at a carboxylate-bridged diiron center, and the catalytic mechanism has been studied intensively (Tinberg & Lippard, 2011). sMMO has a wide substrate range that includes aromatic and heterocyclic compounds (Jiang et al., 2010; Colby et al., 1977). pMMO is composed of three subunits, α, β and γ, also known as pmoB, pmoA, and pmoC, respectively (Zahn & DiSpirito, 1996; Lieberman & Rosenzweig, 2004), arranged in an $\alpha_3\beta_3\gamma_3$ trimer (Lieberman & Rosenzweig, 2005a; Lieberman & Rosenzweig, 2005b). Ammonia monooxygenase (AMO), a pMMO homolog, is the only other enzyme besides pMMO and sMMO known to oxidize methane and is believed to have a similar polypeptide composition as pMMO (Arp et al., 2002; Holmes et al., 1995). The physiological reductant for pMMO has not been identified definitively, but might involve quinones from the quinone pool reduced by a type 2 NADH:quinone oxidoreductase (Choi et al., 2003; Shiemke et al., 2004; Cook & Shiemke, 2002) or by methanol dehydrogenase (Basu et al., 2003). Unlike sMMO, pMMO has a narrow substrate specificity, only oxidizing alkanes containing up to five carbons and alkenes containing up to four carbons (Jiang et al., 2010; Burrows et al., 1984; Smith & Dalton, 1989; Nguyen et al., 1996). The nature of the pMMO metal active site has been controversial (Chan et al., 2004; Lieberman & Rosenzweig, 2004; Balasubramanian & Rosenzweig, 2007; Rosenzweig, 2008; Chan & Yu, 2008; Semrau et al., 2010), and is critically important since pMMO is the predominant methane oxidation catalyst in nature, although it should be noted that other systems oxidize methane in anerobic environments (Thauer, 2011). Major advances toward understanding the pMMO active site have been made over the past few years and are the focus of this review.

Structure of pMMO

Overall architecture

Crystal structures of pMMOs from three different organisms have been determined, the *M. capsulatus* (Bath) pMMO to 2.8 Å resolution (Lieberman & Rosenzweig, 2005a; Lieberman & Rosenzweig, 2005b), the *M. trichosporium* OB3b pMMO to 3.9 Å resolution (Hakemian et al., 2008), and the *Methylocystis* sp. strain M pMMO to 2.68 Å resolution (Smith et al., 2011b). Of the three, the *Methylocystis* sp. strain M pMMO structure is the best quality model (Smith et al., 2011b). The overall architecture of pMMO is the same in all three structures, an $\alpha_3\beta_3\gamma_3$ trimer with three copies each of the pmoB, pmoA, and pmoC subunits. The 100 kDa complex consisting of one copy each of pmoB, pmoA, and pmoC is typically referred to as the pMMO protomer (Figure 1A). The pmoB subunit consists of an N-terminal cupredoxin domain followed by two transmembrane helices, a long linker region, and a C-terminal cupredoxin domain. These cupredoxin domains, located in the periplasm, are the only soluble domains of pMMO. The pmoA and pmoC subunits are primarily composed of transmembrane helices. The recent determination of the *Methylocystis* sp. strain M pMMO structure revealed several discrepancies in these chains with the original *M. capsulatus* (Bath) pMMO crystallographic model, and the *M. capsulatus* (Bath) pMMO structure was re-refined accordingly, leading to a much improved structure (Smith et al., 2011b).

Both the *M. trichosporium* OB3b (Hakemian et al., 2008) and *Methylocystis* sp. strain M (Smith et al., 2011b) pMMO models include an additional transmembrane helix, not found in the *M. capsulatus* (Bath) structure, modeled as polyalanine, adjacent to the pmoC subunit (Figure 1A). The electron density maps and chain tracings clearly indicate that this helix is not a part of either pmoC or pmoA, but the limited resolution has precluded assigning the side chains. This helix is definitely not present in the *M. capsulatus* (Bath) pMMO structure (Figure 1B) and thus may be specific to pMMO from the type II methanotrophs. Unidentified, exogenous helices have been observed other membrane protein structures (Buschmann et al., 2010), and the significance of this helix for pMMO remains unclear.

Crystallographic metal centers

Three metal centers were observed in the original *M. capsulatus* (Bath) pMMO structure and identified using anomalous difference electron density maps (Lieberman & Rosenzweig, 2005a; Sommerhalter et al., 2005). Two copper centers were modeled in the soluble regions of pmoB, a mononuclear site at the interface between the two cupredoxin domains and a dinuclear site close to the membrane interface (Figure 1B). The mononuclear site is coordinated by His 48 and His 72 (Figure 2). The residue equivalent to His 48 is an asparagine in both the *M. trichosporium* OB3b and *Methylocystis* sp. strain M pMMOs, and neither contains copper at this site (Hakemian et al., 2008; Smith et al., 2011b) (Figures 1A, 2). The dinuclear site is coordinated by His 33, His 137, and His 139. Residue His 33 is the amino terminal residue of pmoB (the first 32 residues are a signaling sequence that is removed in vivo), and its amino group is within coordinating distance of the copper (Figure 2). These three histidine residues are conserved in all methanotroph pmoB sequences except those from the Verrucomicrobia (Op den Camp et al., 2009) (Figure 3). Since the crystallographic resolution is not sufficient to distinguish two copper ions so close together, this model relies heavily on extended X-ray absorption fine structure (EXAFS) data indicating the presence of a Cu-Cu interaction at 2.5–2.6 Å (Lieberman et al., 2006; Lieberman et al., 2003). This model is also consistent with the stoichiometry of 2–3 copper ions per 100 kDa *M. capsulatus* (Bath) pMMO protomer reported by several laboratories (Lieberman & Rosenzweig, 2004; Lieberman et al., 2003; Basu et al., 2003). However, in the structure of *Methylocystis* sp. strain M pMMO, two of the three putative dinuclear sites were modeled as mononuclear (Smith et al., 2011b) (Figure 2). This may be due to lability of the copper sites observed during isolation of this pMMO.

The third metal center is located within the membrane and is occupied by zinc in the *M. capsulatus* (Bath) and *Methylocystis* sp. strain M pMMO structures. This zinc is derived from the crystallization buffer and was originally modeled as coordinated by residues Asp 156, His 160, and His 173 from pmoC and Glu 195 from pmoA (Lieberman & Rosenzweig, 2005a). The revised model based on the *Methylocystis* sp. strain M pMMO structure resulted in the replacement of Glu 195 from pmoA with a solvent molecule and increased exposure of this site in the center of the pMMO trimer (Smith et al., 2011b) (Figure 2). In the case of *M. trichosporium* OB3b pMMO, zinc was not required for crystallization, and this site is occupied by copper (Hakemian et al., 2008). It is currently unknown whether the copper at this site is biologically relevant or originates from the addition of copper during the protein purification. The three ligands are highly conserved, even in the Verrucomicrobia sequences (Hakemian & Rosenzweig, 2007; Op den Camp et al., 2009) (Figure 3).

The pMMO active site

Metal content and location

A variety of models for the pMMO active site have been proposed in the context of the crystallographic data (Rosenzweig, 2008). One model involves the presence of a trinuclear copper center, first suggested on the basis of electron paramagnetic resonance (EPR) spectroscopic data (Chen et al., 2004; Nguyen et al., 1996). Although not present in the crystal structures, this trinuclear copper center is suggested to reside at a hydrophilic patch of residues within the pmoA and pmoC subunits (Chan et al., 2007) (Figure 1B). This model also includes approximately ten additional copper sites within the C-terminal cupredoxin domain of pmoB (Yu et al., 2007), which are not observed in the crystal structure. A second model invokes iron as the active site metal. On the basis of Mössbauer spectroscopic data, a diiron center similar to that in sMMO was proposed to be present in pMMO at the site of the crystallographic zinc ion (Martinho et al., 2007) (Figure 1). Finally, the consistent observation of copper at the modeled dinuclear site has led to the suggestion that the catalytic site is at this location within the pmoB subunit (Rosenzweig, 2008).

These models were tested experimentally by removing all the metal from membrane-bound *M. capsulatus* (Bath) pMMO using cyanide and then assessing which metal ions restore both methane oxidation and propylene epoxidation activity (Smith et al., 2011a). Up to 90% of enzyme activity was recovered upon the addition of 2–3 copper ions per 100 kDa pMMO protomer. By contrast, iron addition has no effect on pMMO activity (Balasubramanian et al., 2010). These observations are consistent with a copper active site model, and similar results were obtained with *Methylocystis* sp. strain M pMMO, although this pMMO has much lower activity upon isolation (Smith et al., 2011b). The addition of more than 2–3 equivalents of copper results in reversible inhibition of activity (Balasubramanian et al., 2010), which can be ameliorated by the addition of catalase, and may result from hydrogen peroxide formation (Miyaji et al., 2009).

To localize the active site within the pMMO structure, constructs were generated corresponding to the two soluble cupredoxin domains of *M. capsulatus* (Bath) pmoB, both alone (spmoBd1 and spmoBd2) and connected (spmoB) via a synthetic linker that replaces the two transmembrane helices (Balasubramanian et al., 2010; Smith et al., 2011a) (Figure 1B). These proteins, which express in *E. coli* primarily as inclusion bodies, were refolded in the presence of copper, and their copper binding, spectroscopic, and activity properties were analyzed. Both spmoB and spmoBd1 bind copper, but spmoBd2 does not, contrary to previous reports (Yu et al., 2007). Methane oxidation activity is also observed for both spmoB and spmoBd1, strongly suggesting that the active site is housed within this soluble part of pmoB (Balasubramanian et al., 2010). To pinpoint the active site location, site directed variants designed to disrupt the copper sites were prepared. Activity is observed upon disruption of the mononuclear site (spmoB_H48N), but is abolished in variants that interfere with the dicopper site (spmoB_H137,139A) or both sites (spmoB_H48N_H137,139A) (Balasubramanian et al., 2010). These results suggest that the active site of pMMO is the crystallographically modeled dicopper site (Balasubramanian et al., 2010).

The dicopper active site model

There are several unresolved issues with the assignment of the crystallographic dicopper center as the pMMO active site. First, the three histidine ligands are not conserved in the recently discovered Verrucomicrobia pmoB sequences (Op den Camp et al., 2009) (Figure 3), suggesting that a different type of metal center would have to be present in these pmoB subunits. The Verrucomicrobia sequences are overall quite divergent from the

Proteobacterial sequences (although the ligands to the crystallographic zinc site are conserved) and are evolutionarily distant (Op den Camp et al., 2009). It is perhaps not surprising that there is some divergence given that these methanotrophs exist in high temperature environments with pH values ranging from 1–4.5. Although it is not clear what pH the active site is exposed to, it is worth noting that at pH 4.5, methionine has a higher affinity for Cu(I) than histidine (Rubino et al., 2011). Thus, it is conceivable that the extreme environment may necessitate a completely different metal active site.

Another issue is the low activity of the spmoB proteins. The methane oxidation activity is about 10% of that of intact membrane-bound pMMO (Table 1) (Balasubramanian et al., 2010), which is in turn about 10% of whole cell activity (Martinho et al., 2007). This observation could imply that the active site is not within this subunit, but it is unlikely that artifactual methane oxidation would be observed. It may be that the more exposed nature of the site in spmoB, which is also probably only partially loaded with copper, along with inherent problems in refolding proteins from inclusion bodies (Singh & Panda, 2005), account for the low activity. Moreover, the functional roles of the membrane domains have not been elucidated and their absence likely impacts the activity of spmoB. One possibility is that methane enters the active site from the membrane, where it would be more soluble (Miller et al., 1977), and this more favorable and defined substrate entryway is not present in the soluble domain.

Finally, the nuclearity of the active site has not been established definitively. EXAFS data for the three pMMOs studied to date indicate the presence of a short Cu-Cu interaction at ~2.5 Å, which increases to ~2.6 Å upon chemical reduction (Hakemian et al., 2008; Lieberman et al., 2006; Lieberman et al., 2003; Smith et al., 2011b). The only location in the crystal structure consistent with a multinuclear copper site is the dicopper center modeled in the pmoB subunit, but the current resolution of the structures (2.7 Å at best) precludes clearly resolving two copper ions separated by < 2.7 Å. This site is strongly occupied with copper in all pMMO structures and can be modeled as dinuclear (Lieberman & Rosenzweig, 2005a) or, in some subunits of the *Methylocystis* sp. strain M pMMO (Smith et al., 2011b), as mononuclear (Figure 2). The only visible ligands are the three histidine side chains and the amino terminus of pmoB, leaving open the question of whether exogenous bridging or terminal ligands might also be present. The copper binding stoichiometry of *M. capsulatus* (Bath) pMMO of 2–3 copper ions per protomer (Lieberman & Rosenzweig, 2004) (Table 1) is consistent with the presence of mononuclear and dinuclear sites, assuming that the crystallographic zinc site is not occupied with copper. The copper binding stoichiometries for *M. trichosporium* OB3b and *Methylocystis* sp. strain M pMMO are closer to 2 copper ions per protomer (Hakemian et al., 2008; Smith et al., 2011b) (Table 1), which could be interpreted as a dicopper center in pmoB or as a monocopper center in pmoB and a monocopper center in the membrane, as observed in the *M. trichosporium* OB3b pMMO structure (Hakemian et al., 2008). However, the copper occupancy of this intramembrane site is quite low and may not account for the measured stoichiometry. Moreover, the metal stoichiometry observed in crystal structures is often not consistent with that measured for purified proteins in solution (Sommerhalter et al., 2005). Also relevant is the reduced activity of the *M. trichosporium* OB3b and *Methylocystis* sp. strain M pMMOs in comparison to *M. capsulatus* (Bath) pMMO (Hakemian et al., 2008; Smith et al., 2011b) (Table 1), possibly because of increased lability of the copper active site. Thus, although most of the data, including oxygen binding experiments (vide infra) are compatible with the dicopper active site model, a high resolution structure of the site is essential to ascertain the nuclearity.

Substrate and product binding

Despite the availability of crystallographic data and strong evidence for the active site being located in the pmoB subunit, direct evidence for methane or methanol binding sites and pathways to access the active site has not been obtained. Acetylene has long been known to act as a suicide substrate of pMMO, and studies using ^{14}C-labeled acetylene suggest that the label ends up in the pmoA subunit (Cook & Shiemke, 1996; Zahn & DiSpirito, 1996) and might also bind to the pmoB subunit (Zahn & DiSpirito, 1996). Similar experiments with AMO also resulted in labeling of the pmoA subunit (Hyman & Wood, 1985; Hyman & Arp, 1992), specifically residue His 191, and this residue is assumed to be part of or neighboring the active site (Gilch et al., 2009). The analogous residue in *M. capsulatus* (Bath) pMMO is Tyr 186, located ~14 Å from the dicopper site in pmoB, ~24 Å from the hydrophilic patch that is proposed to house the tricopper site, and ~26 Å from the zinc site proposed to house the diiron active site. It should also be noted that the retracing of pmoA and pmoC in the *Methylocystis* sp. strain M pMMO structure places the zinc site within pmoC (Smith et al., 2011b), and if it were to house a diiron active site as suggested (Martinho et al., 2007), pmoC, not pmoA, should be labeled. It thus remains unclear how to interpret the acetylene binding data in the context of the structural and other biochemical data.

Substrate and product binding have also been explored computationally. Using Global Protein Surface Survey (GPSS) analysis, a hydrophobic pocket adjacent to the proposed tricopper center was identified in *M. capsulatus* (Bath) pMMO. This pocket is lined with Phe 50, Trp 51, and Trp 54 from pmoA, and is suggested to accommodate hydrocarbons up to five carbons in length (Ng et al., 2008). Recent work on *M. trichosporium* OB3b pMMO using the substrates n-butane and n-pentane indicates that the (R)-2-alcohol product is formed enantioselectively (Miyaji et al., 2011), consistent with previous studies of *M. capsulatus* (Bath) pMMO (Elliott et al., 1997). Therefore, the binding cavity can orient these substrates. Since pMMO can oxidize pentane, but not hexane, the volume of the substrate binding site can be approximated to ~138 $Å^3$, and the existence of distinct binding sites for methane and larger substrates has been suggested (Miyaji et al., 2011). Little is known about product egress, but cryoelectron microscopic studies suggest that methanol dehydrogenase might interact with the periplasmic soluble domains of pmoB (Myronova et al., 2006), and it is plausible that methanol would exit the enzyme through these regions.

Oxygen binding

The O_2 reactivity of the copper sites in pMMO has been addressed computationally (Shiota & Yoshizawa, 2009; Yoshizawa & Shiota, 2006; Chan et al., 2004). According to DFT and QM/MM calculations using the two copper centers modeled in the pmoB subunit of the *M. capsulatus* (Bath) structure, both a mononuclear Cu^{III}-oxo species and a dinuclear mixed valent bis(μ-oxo)$Cu^{II}Cu^{III}$ species are sufficiently reactive to oxidize methane, although formation of the dinuclear species is energetically more favorable than the mononuclear species (Shiota & Yoshizawa, 2009; Yoshizawa & Shiota, 2006). Related to the potential ability of a dicopper species to oxidize methane, the active species in a Cu-ZSM-5 zeolite that converts methane to methanol has been shown to be a mono-μ-oxo Cu^{II}_2 species that forms from a μ-η^2:η^2-peroxo Cu^{II}_2 precursor (Groothaert et al., 2005; Vanelderen et al., 2011; Woertink et al., 2009; Smeets et al., 2010).

The binding of O_2 to pMMO has also been investigated experimentally. Recent data show that an optical feature at 345 nm forms upon reaction of reduced, solubilized *M. capsulatus* (Bath) pMMO with H_2O_2, and the same feature can also be observed upon reaction of reduced spmoB with either H_2O_2 or O_2 (Culpepper & Rosenzweig, 2012) (Figure 4). The energy and intensity of this band are similar to that of the well characterized μ-η^2:η^2-peroxo Cu^{II}_2 species of hemocyanin and tyrosinase (Solomon et al., 1996; Solomon et al., 2011).

The feature may alternatively correspond to a met Cu^{II}_2 species, like that of hemocyanin (Zlateva et al., 1998; Andrew et al., 1993), or to a hydroxo bridged Cu^{II}_2 species, similar to the type 3 site of multicopper oxidases (Solomon et al., 1996). Sample constraints, including the instability of spmoB and the presence of heme contaminants in pMMO, have precluded further analysis of this species by resonance Raman spectroscopy, however. Importantly, the 345 nm optical feature is also observed for the spmoB_H48N variant, but not for the two variants (spmoB_H137,139A and spmoB_H48N_H137,139A) that disrupt the modeled dicopper center, suggesting that O_2 is indeed binding at the proposed active site. Moreover, disappearance of the optical feature is enhanced by the presence of CH_4, consistent with it possibly being on the reaction pathway (Culpepper & Rosenzweig, 2012). These data support the assignment of the active site and are consistent with the presence of a dicopper center. The parallel to the Cu-ZSM-5 zeolite is also striking and reinforces the plausibility of the dicopper active site model.

Outlook

Since the last *Critical Review* on pMMO (Lieberman & Rosenzweig, 2004), much progress has been made toward understanding the molecular details of methane oxidation. Three crystal structures of pMMO have been determined, revealing the overall architecture and the locations of several metal centers. Biochemical data indicate that pMMO activity is copper-dependent, and the active site has been localized to the crystallographically modeled dicopper center in the pmoB subunit. Initial spectroscopic evidence for O_2 binding at this dicopper site has also been obtained. Taken together, these data are consistent with the active site of pMMO being a dicopper center. Concerns about absence of the ligands in the Verrucomicrobia pmoB sequences and the site nuclearity persist, however. High resolution crystallographic data are absolutely critical to further characterization of the copper active site. The development of the soluble, recombinant spmoB proteins represents the first opportunity to probe pMMO function by site-directed mutagenesis, and suggests that optimized soluble pMMO fragments might be a viable approach toward obtaining additional crystallographic, spectroscopic, and mechanistic data. The function of the transmembrane regions has not been elucidated and will be an important direction for future work. In this regard, the ability to express intact pMMO would be highly desirable. Overall, a multipronged strategy involving pMMO, recombinant models, and studies of related synthetic systems will be required to resolve atomic details of the active site and proceed to the next stages of investigating the catalytic mechanism.

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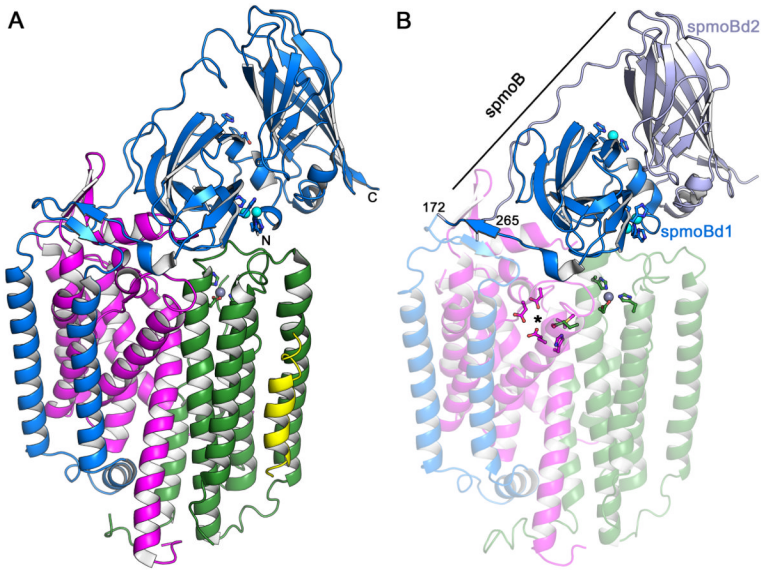

Figure 1.

Overall architecture of pMMO. (A) Structure of *Methylocystis* sp. strain M pMMO protomer (PDB accession code 3RGR). The pmoB, pmoA, and pmoC subunits are shown in blue, magenta, and green, respectively. The N- and C-termini of pmoB are labeled. An exogenous helix is shown in yellow. Copper ions are shown as cyan spheres and a zinc ion is shown as a gray sphere. Ligands are shown as ball-and-stick representations. (B) Structure of *M. capsulatus* (Bath) pMMO protomer (PDB accession code 3RGB). The amino terminal domain of pmoB (spmoBd1) is shown in blue, the carboxy terminal domain of pmoB is shown in gray (spmoBd2), and the two transmembrane helices are shown in transparent blue. In the recombinant spmoB protein, spmoBd1 and spmoBd2 are linked by a GKLGGG sequence connecting residues 172 and 265 (labeled). The pmoA and pmoC subunits are shown in transparent magenta and transparent green, respectively. A hydrophilic patch of residues proposed to house a tricopper active site is denoted with an asterisk. The mononuclear copper site at the interface of the two spmoB domains is not present in the *Methylocystis* sp. strain M pMMO structure. [A color version of this figure is available online.]

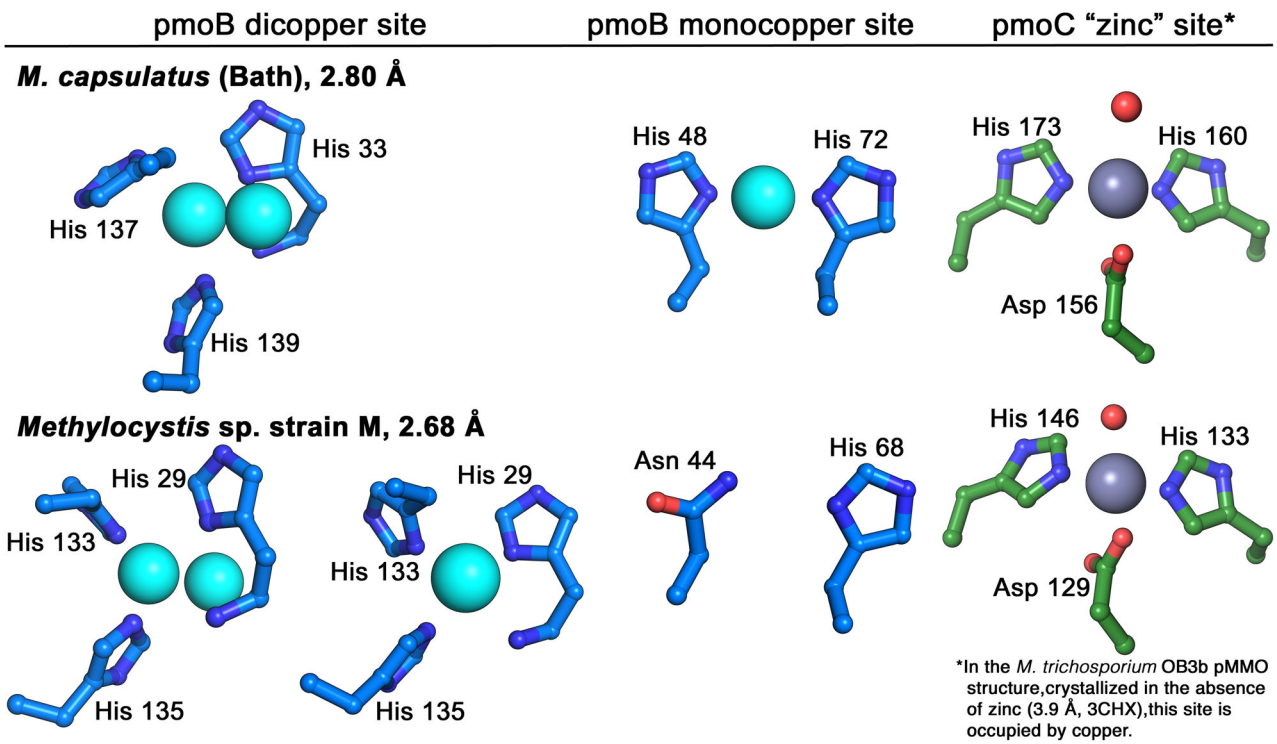

Figure 2.

Metal centers modeled in the pMMO crystal structures. The pmoB site modeled as dicopper in the *M. capsulatus* (Bath) pMMO structure is modeled as dicopper in one protomer and monocopper in two protomers in the *Methylocystis* sp. strain M pMMO structure. The monocopper site in pmoB from *M. capsulatus* (Bath) pMMO does not contain copper in the other pMMO structures. In both structures, zinc is found in the transmembrane site as a result of including zinc in the crystallization buffer. Copper occupies this site in *M. trichosporium* OB3b pMMO. [A color version of this figure is available online.]

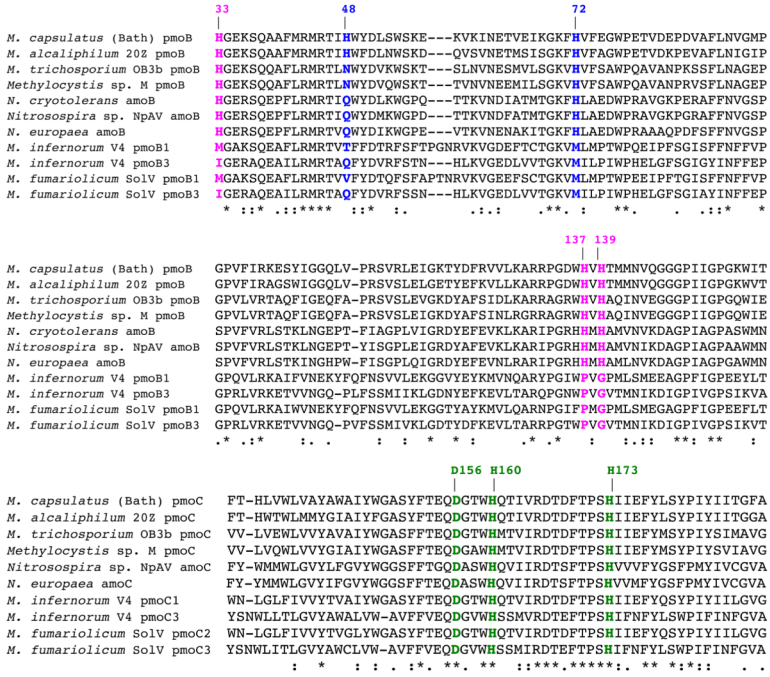

Figure 3.

Multiple sequence alignments of representative pmoB/amoB and pmoC/amoC sequences containing the ligands to the crystallographically observed pMMO metal centers. The ligands to the pmoB dicopper site are shown in magenta, the ligands to the pmoB monocopper site in *M. capsulatus* (Bath) pMMO are shown in blue, and the ligands to the zinc site are shown in green. The ligands to the dicopper center are not conserved in the bottom four Verrucomicrobia sequences. [A color version of this figure is available online.]

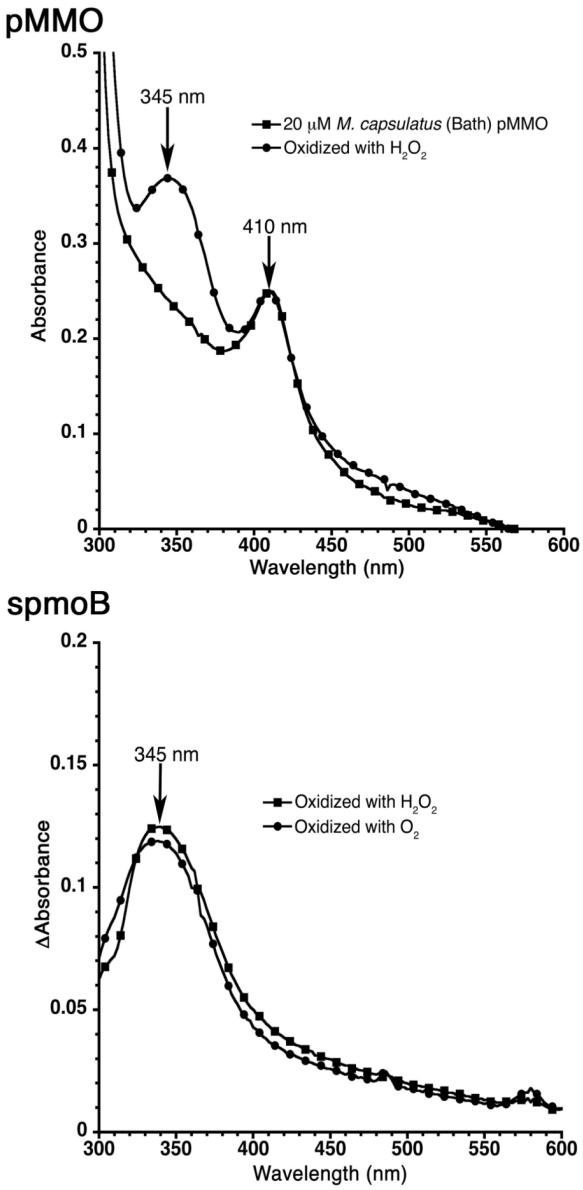

Figure 4.

Optical spectrum of detergent-solubilized pMMO from *M. capsulatus* (Bath) (top) and difference spectra of recombinant spmoB (bottom) reduced and oxidized with either H_2O_2 or O_2. The feature at 345 nm is similar to that observed for the μ-η^2:η^2-peroxo Cu^{II}_2 species formed in hemocyanin and tyrosinase. The peak at 410 nm derives from heme contaminants in the pMMO sample.

Table 1

Copper content and enzyme activity of pMMOs and soluble pmoB fragments.[1,2]

	Membrane-bound		Purified		Reference
	Coppers per 100 kDa protomer	**Activity (nmol propylene oxide $min^{-1}mg^{-1}$)**	**Coppers per 100kDa protomer**	**Activity (nmol propylene oxide $min^{-1}mg^{-1}$)**	
M. capsulatus (Bath)	20.8 ± 4.5	16	2.4 ± 0.4	17.7	Leiberman et al., 2003
M. trichosporium OB3b	4.8 ± 1.1	3.0 ± 05	1.4 ± 0.6	0.11 ± 0.1	Hakemian et al., 2008
Methylocystis sp. strain M	2.29 ± 0.22	5.3 ± 1.4	2.11 ± 0.46[3]	1.24 ± 0.21[3]	Smith et al., 2011b
	Coppers per 100 kDa	Activity (nmol CH_3OH $min^{-1}\mu mol^{-1}$)	Coppers per mole	Activity (nmol CH_3OH $min^{-1}\mu mol^{-1}$)	
M. capsulatus (Bath)	12.4 ± 4	2290 ± 60			Balasubramanian et al., 2010
spmoB			2.84 ± 0.66	203.1 ± 20.2	”
spmoBd1			1.59 ± 0.84	19.3 ± 4.7	”
spmoBd2			0.24 ± 0.04	0	”
spmoB_H48N			1.86 ± 0.52	14.8 ± 9.2	”
spmoB_H137,139A			0.75 ± 0.15	0	”
spmoB_H48N_H137,139A			0.82 ± 0.36	0	”

[1] Data from the Rosenzweig laboratory are summarized. Comprehensive tables can be found in Lieberman & Rosenzweig, 2004 and Semrau et al., 2010.

[2] Activity measured using duroquinol as a reductant.

[3] Copper content and activity values were obtained from detergent solubilized samples.