Biochemistry and Molecular Biology of Methane Monooxygenase

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Tim Nichol, J. Colin Murrell, and Thomas J. Smith

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Abstract

Methane-oxidizing bacteria (methanotrophs) are a unique group of aerobic bacteria that can gain all of their carbon and energy requirements from methane. The enzymes that catalyze the first step in the bacterial methane oxidation pathway, the oxidation of methane to methanol, are called methane monooxygenases. These are remarkable enzymes because methane is chemically very stable, and to convert methane to methanol chemically requires expensive catalysts, high temperatures, and pressures. There are two types of methane monooxygenase that occur in methanotrophs, a membrane-bound, particulate methane monooxygenase, and a cytoplasmic, soluble methane monooxygenase which belongs to a class of enzymes known as soluble diiron monooxygenases. The expression of these enzymes in methanotrophs is often regulated by the availability of copper. The soluble methane monooxygenase has attracted significant attention

T. Nichol (✉) · T. J. Smith
Biomolecular Sciences Research Centre, Sheffield Hallam University, Sheffield, UK
e-mail: t.nichol@shu.ac.uk; T.J.Smith@shu.ac.uk

J. C. Murrell
School of Environmental Sciences, University of East Anglia, Norwich Research Park, Norwich, UK
e-mail: J.C.Murrell@uea.ac.uk

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and has considerable potential in biocatalysis and bioremediation since it can co-oxidize a very wide range of aliphatic and aromatic compounds, even though methanotrophs themselves do not grow on these compounds. We review here the biochemistry and molecular biology of both the particulate and soluble methane monooxygenases and their biotechnological potential.

1 Introduction

Methane-oxidizing bacteria (methanotrophs) are remarkable in being able to use the inert methane molecule to provide all of the chemical energy for the cell and also to synthesize the carbon building blocks for all of the macromolecules in the cell. They carry out the oxidation of methane via the enzyme methane monooxygenase (MMO) and subsequently use the same enzymes found in other aerobic Gram-negative methylotrophic bacteria for further oxidation of methanol to formaldehyde, formate, and carbon dioxide and for assimilation of carbon, at the oxidation level of formaldehyde, into cellular constituents (Fig. 1) (Anthony 1982; Dalton 2005; Hanson and Hanson 1996; Trotsenko and Murrell 2008; Lawton and Rosenzweig 2016).

In methanotrophs there are two structurally and biochemically distinct forms of MMO, particulate methane monooxygenase (pMMO) and soluble methane monooxygenase (sMMO), which oxidize methane to methanol. pMMO is a

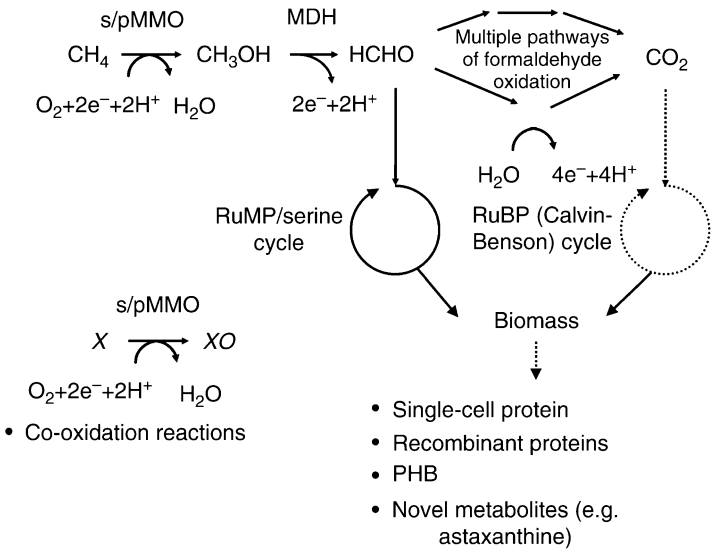

Fig. 1 Principal metabolic pathways of methanotrophic metabolism showing the roles of soluble and particulate methane monooxygenase (sMMO/pMMO), methanol dehydrogenase (MDH), and the cyclical pathways for carbon fixation. Biotechnologically important reactions and products discussed in the text are shown as bullet points

copper-containing enzyme that is associated with unusual intracellular membranes found in type I methanotrophs as vesicular disks arranged in bundles throughout the cell and as paired peripheral layers in type II methanotrophs. sMMO is a cytoplasmic non-heme iron enzyme complex. The best characterized methanotrophs, *Methylococcus capsulatus* (Bath) (type I) and *Methylosinus trichosporium* OB3b (type II), can produce either form of MMO (reviewed in Murrell et al. 2000). The principal factor known to govern expression of the two types of MMO in these organisms is the concentration of available copper, and copper homeostasis is carefully regulated within methanotrophs through copper uptake systems and copper storage mechanisms (Vita et al. 2015, 2016; Gu and Semrau 2017). At high copper-to-biomass ratio, pMMO is produced, whereas the soluble form of the enzyme is expressed only when the copper-to-biomass ratio during growth is low (Stanley et al. 1983). Many methanotrophs such as the type I methanotrophs *Methylomonas methanica* and *Methylomicrobium album* BG8 possess only pMMO, and previously the dogma was that all methanotrophs contained pMMO. More recently, however, the facultative type II methanotroph *Methylocella silvestris* and obligate methanotroph *Methyloferula stellata* have been shown to possess only the sMMO system and do not possess pMMO (Dedysh et al. 2005; Theisen et al. 2005; Vorobev et al. 2011; Crombie and Murrell 2014; Dedysh et al. 2015). While the majority of methanotrophs are aerobic, *Methylomirabilis oxyfera* appears to grow anaerobically through oxidation of methane via pMMO using O_2 generated in situ from nitrite (Welte et al. 2016). The two families of MMOs share no detectable similarity in amino acid sequence or three-dimensional structure and are not evolutionarily related. It may be because methane is such a small and unfunctionalized substrate that both sMMO and pMMO are able to co-oxidize a range of hydrocarbons and chlorinated pollutants in addition to their natural substrate. Hence sMMO and pMMO have biotechnological potential that extends far beyond their ability to oxidize methane to methanol (see later).

2 Biochemistry of Particulate Methane Monooxygenase (pMMO)

pMMO is a copper-containing, membrane-associated enzyme (Nguyen et al. 1998; Smith and Dalton 1989; Zahn and DiSpirito 1996; Ross and Rosenzweig 2017), and molecular ecology studies indicate that pMMO is probably responsible for most of the oxidation of methane carried out by aerobic methanotrophs in the environment (reviewed in McDonald et al. 2008). Being a membrane protein, the biochemistry of pMMO has lagged behind that of sMMO largely due to problems in solubilizing the pMMO away from membranes and purifying it in active form. The use of dodecyl-β-D-maltoside as detergent (Smith and Dalton 1989), however, allows recovery of activity after solubilization, and subsequent development of purification protocols has allowed the enzyme to be purified in an active form. Active preparations of pMMO generally contain three polypeptides, of about 49, 27, and 22 kDa. The 27-kDa subunit can be labeled by the inhibitor acetylene (a suicide substrate for both pMMO and sMMO), and previously it was thought that the active site resided on this subunit.

More recent structural studies suggest, however, that the active site may reside on the 49-kDa subunit or indeed may be shared between more than one subunit (reviewed in Hakemian and Rosenzweig 2007, Balasubramanian et al. 2010). The 49-, 27-, and 22-kDa components are encoded by the genes *pmoB*, *pmoA*, and *pmoC*, respectively, which are multicopy genes (see below) that are induced in response to growth of methanotrophs at high copper-to-biomass ratio. The crystal structure of pMMO, albeit of protein of rather low activity, showed that the enzyme has an $(\alpha\beta\gamma)_3$ stoichiometry and gave the first indication of the atomic resolution structure of the enzyme (Lieberman and Rosenzweig 2005). Single particle analysis and associated biochemical studies have indicated that native pMMO forms a complex with methanol dehydrogenase, which may supply electrons to the enzyme (Kitmitto et al. 2005; Myronova et al. 2006). While all active preparations of pMMO contain copper, the numbers and roles of copper ions in the active form of the enzyme continue to be debated, and it has also been suggested that iron plays a role in pMMO (Martinho et al. 2007 reviewed in Hakemian and Rosenzweig 2007; Semrau et al. 2010; Ross and Rosenzweig 2017). Recent quantum refinement of the crystal structure data suggests a mononuclear copper center in the crystallized form of the protein (Cao et al. 2018).

An expression system for pMMO has been developed within *Escherichia coli*, which is capable of producing active PmoB, capable of methane oxidation (Balasubramanian et al. 2010). This has allowed further insights into the nature of the active site and enables future site-directed mutagenesis studies to elucidate the exact catalytic mechanism. Studies using protein refolding of truncated recombinant PmoB with metal ions suggest that only copper is required for catalysis and the addition of iron does not restore or increase activity (Balasubramanian et al. 2010). Little is currently known about the catalytic cycle of pMMO. Retention of stereochemistry is observed during oxygenation of certain chiral alkanes, and so the mechanism of C-H bond breakage is likely to be concerted (rather than involving radical or cation intermediates). It will be interesting to see what similarities there are between the catalytic mechanism of pMMO and sMMO, which catalyze the same reaction within such different enzyme environments.

The substrate profile of pMMO is very much narrower than that of sMMO. pMMO oxidizes methane and linear short-chain hydrocarbons but not aromatic compounds, the alicyclic hydrocarbon cyclohexane or the branched aliphatic 2-methylpropane, all of which are substrates of sMMO (reviewed in Smith and Dalton 2004). Thus it seems that access to the active site of pMMO is sterically more restricted than in the soluble enzyme. Consistent with this, acetylene is a potent suicide substrate of both pMMO and sMMO, whereas the larger phenylacetylene molecule is much more effective against sMMO (Lontoh et al. 2008).

3 Biochemistry of Soluble Methane Monooxygenase (sMMO)

sMMO is a three-component binuclear iron active center monooxygenase that belongs to a large group of bacterial hydrocarbon oxygenases (reviewed in Leahy et al. 2003) known as the soluble diiron monooxygenases (SDIMOs) (Coleman et al.

2006; Nichol et al. 2015; Trehoux et al. 2016), which are also homologous to the R2 subunit of class I ribonucleotide reductase. sMMO is currently the better characterized form of MMO since it is more easily purified than the particulate enzyme. More is known about the molecular mechanisms regulating expression of sMMO, and a system for expression of recombinant sMMO, a prerequisite for site-directed mutagenesis studies, has also been developed (Smith et al. 2002).

The most well-characterized sMMO systems are from *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b. sMMO, encoded by a six-gene operon *mmoXYBZDC*, has three main components: (1) a 250-kDa hydroxylase with an $(\alpha\beta\gamma)_2$ structure (encoded by *mmoX*, *mmoY*, and mmo*Z*, respectively) – MmoX contains the binuclear iron active center where substrate oxygenation occurs; (2) a 39-kDa NAD(P)H-dependent reductase (MmoC) with flavin adenine dinucleotide (FAD) and Fe_2S_2 prosthetic groups; (3) a 16-kDa component (MmoB) known as protein B or the coupling/gating protein that does not contain prosthetic groups or metal ions (Fig. 2) (Smith and Dalton 2004; Smith and Murrell 2008; Sazinsky and Lippard 2015; Sirajuddin and Rosenzweig 2015; Lee 2016). The 12-kDa component MmoD works in conjunction with the chalkophore methanobactin to regulate the expression of sMMO during low copper concentration (Semrau et al. 2013, 2018; DiSpirito et al. 2016). There are X-ray crystal structures for the hydroxylase component (Elango et al. 1997; Rosenzweig et al. 1993), NMR-derived structures for protein B (Walters et al. 1999), and NMR structural data for the flavin domain of the reductase (Chatwood et al. 2004). The complex formed by the three components has been studied structurally via small angle X-ray scattering analysis and biophysically by electron paramagnetic resonance, ultracentrifugation, and calorimetric analysis (reviewed in Hakemian and Rosenzweig 2007). Crystallography of the complex formed between the hydroxylase and protein B (Fig. 3) (Lee et al. 2013) indicates that binding of protein B induces changes in hydroxylase conformation that may allow substrate entry and product egress. They may also facilitate proton transfer required by the catalytic cycle.

The catalytic cycle of sMMO has been extensively studied, and excellent progress has been made toward understanding the mechanism of oxygen and

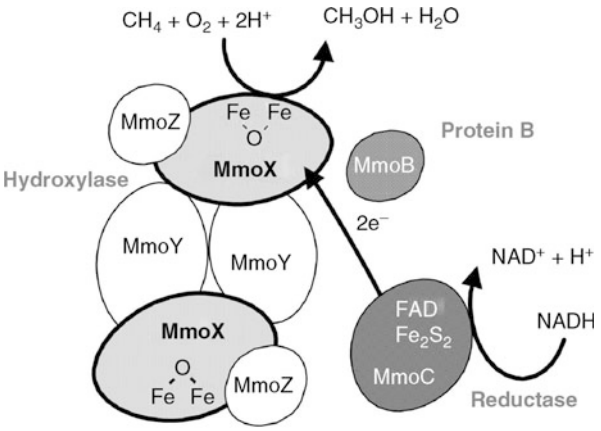

Fig. 2 Schematic of the sMMO enzyme complex

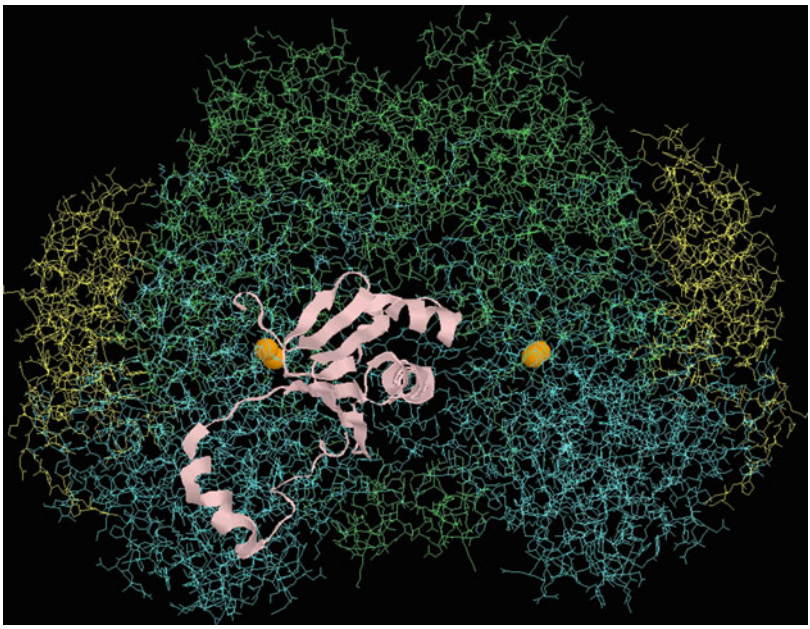

Fig. 3 Structure of the hydroxylase component of sMMO of *Mc. capsulatus* (Bath). The α, β, and γ subunits are shown in blue, green, and yellow, respectively. The iron atoms of the diiron centers are shown as orange spheres. Protein B is shown as a pink ribbon diagram at its binding position to the α and β subunits

hydrocarbon activation at the binuclear iron center. More detailed reviews and descriptions of the intermediates that are known in the catalytic cycle of sMMO can be found elsewhere (Baik et al. 2003; Smith and Dalton 2004; Sazinsky and Lippard 2015; Ross and Rosenzweig 2017). In order to comprehend the remarkable ability of sMMO to oxidize the unreactive methane molecule, the most noteworthy intermediate is the so-called compound Q. Compound Q accumulates when the reduced (Fe^{II}-Fe^{II}) hydroxylase is reacted with O_2 in the presence of protein B. The active center of compound Q is in a high-valent diferryl (Fe^{IV}-Fe^{IV}) state (Banerjee et al. 2015). It may have a six-membered ring-bridged structure rather than the four-membered ring "diamond core" structure proposed previously (Castillo et al. 2017). In the absence of oxidizable substrates, compound Q is astonishingly stable ($t_{1/2} \approx 14$ s in aqueous solution at 4 °C); however, this intermediate rapidly oxidizes methane and other substrates and is kinetically competent, i.e., the oxidation rate observed is high enough to account for the rate seen during steady-state catalysis. The mechanism via which sMMO breaks the unreactive C-H bond of methane continues to be intensely debated (as reviewed in Baik et al. 2003; Hakemian and Rosenzweig 2007; Jin and Lipscomb 2000; Jasniewski and Que 2018). Radical, ionic, and concerted mechanisms have been suggested. Evidence from the use of radical clock substrates and theoretical studies suggests a reaction with multiple pathways and the possible involvement of a captive substrate-derived

radical species (Sazinsky and Lippard 2015). Results using stopped-flow spectroscopy have established the involvement of quantum mechanical tunneling of hydrogen nuclei in breaking the C-H bond of methane (Zheng and Lipscomb 2006; Tinberg and Lippard 2010).

The active site pocket of sMMO is a hydrophobic cavity deeply buried in the protein, which has been shown by molecular docking studies to be the energetically most favorable place for binding of methane and other small substrates, and clearly substrates as large as di-aromatics must be able to gain access to this cavity and the adjacent binuclear iron center (Zhang et al. 2017). The side chain of residue Leu 110 in the α-subunit of the hydroxylase partly blocks the aperture between the substrate-binding pocket and the innermost of a chain of cavities that communicate between the active center and the outside and may form the route for substrate entry and product exit. Leu 110 exhibits different conformations in different crystal forms of the enzyme. In the "closed" conformation, it blocks access to the active site, while in the "open" conformation, it permits a 2.6-Å diameter channel into the substrate-binding cavity. A larger conformational change, which may be caused by interaction with the other components of the sMMO complex, could open this "leucine gate" further, to allow passage of substrates and products (Rosenzweig et al. 1997). Site-directed mutagenesis studies have indicated that Leu 110 is important in determining the precision with which aromatic substrates can be oriented in the active site but is not the limiting factor on the size of substrate that can enter (Borodina et al. 2007; Sigdel et al. 2015). Recently crystal structures of MmoB bound MmoH have indicated a change in the conformation of Phe 188 upon binding of MmoB. This suggests that Leu 110 and Phe 188 conformations, mediated by MmoB binding, have a role in controlling substrate access to the active site (Lee et al. 2013). At the time of writing, a study made available as a preprint (Cho et al. 2018) reports crystallographic data showing that binding of MmoD to the hydroxylase also opens this potential substrate access route, although the crystal structure data are not currently available.

While much remains to be discovered about the molecular mechanism of substrate recognition and oxidation by sMMO, it is clear that this enzyme produces in its active site one of the most powerful oxidizing agents in nature and has a substrate-binding pocket that can accommodate a wide range of oxidation substrates in addition to the natural substrate methane. Recent advances in understanding the interaction between the sMMO components may inform future mutagenesis studies to more effectively manipulate the selectivity and catalytic properties of the enzyme.

4 Molecular Biology and Regulation of Methane Monooxygenases

In the chromosome of *Mc. capsulatus* Bath, there are two copies of the pMMO gene cluster *pmoCAB* and an additional copy of *pmo*C (Stolyar et al. 2001). Duplication of the homologous genes *amoCAB*, encoding the ammonia monooxygenase (AMO) in nitrifying bacteria, has also been observed, and it has been suggested that both

pMMO and AMO enzymes may be evolutionarily related. A high degree of homology of pMMOs (80–94%) and duplication of *pmo*CAB genes also occurs in the type II methanotrophs *Ms. trichosporium* and *Methylocystis*. Type II methanotrophs with very different *pmoA* genes have also been found: conventional *pmoA* or *pmoA1* and novel *pmoA* or *pmoA*2 (Tchawa Yimga et al. 2003). In *Methylocystis* strain SC2 *pmoA1* and *pmoA2* gene copies are each part of a complete *pmoCAB* gene cluster (*pmoCAB1* and *pmoCAB2*) which exhibit low levels of identity at both the DNA level (67.4–70.9%) and the derived protein level (59.3–65.6%), but the secondary structures predicted for PmoCAB1 and PmoCAB2, as well as the derived transmembrane-spanning regions, are nearly identical (Ricke et al. 2004). The conventional pMMO genes encode a pMMO that is expressed and oxidizes methane only at high concentrations (>600 ppmv), whereas *pmoCAB*2 encoding the more unusual isoenzyme pMMO2 is constitutively expressed and oxidizes methane at low concentrations, even at the trace levels of atmospheric methane (2 ppmv) (Baani and Liesack 2008). This may well be the MMO enzyme system present in soils which have been observed to be dominated by type II methanotrophs and which oxidize methane at atmospheric concentrations.

In *Mc. capsulatus* Bath, six ORFs organized in one operon *mmo*XYBZDC encode the structural genes for sMMO. The exact mechanism of reciprocal regulation of sMMO and pMMO synthesis by Cu ions is not known. Transcription of the *mmo* operon is initiated from a σ^{n}-(σ^{54})-dependent promoter which requires a transcriptional activator for the formation of an active transcriptional complex. Located near the structural genes in the sMMO gene cluster of *Mc. capsulatus* Bath and *Ms. trichosporium* OB3b are two additional genes *mmo*R and *mmo*G. MmoR encoded by *mmo*R belongs to a class of transcriptional activators which enhance binding of RNA polymerase σ^{N} (RpoN) to promoters which are regulated by this alternative σ factor. MmoG is a homologue of the chaperonin GroEL and may be required for assembly of MmoR or indeed for assembly of the sMMO complex itself (Csaki et al. 2003; Stafford et al. 2003). Mutagenesis of *mmoR*, *mmoG*, or *rpoN* in these methanotrophs prevents expression of sMMO. Recently two copies of *mmoX* have been observed in *Methylosinus sporium* 5; however, mutagenesis of the second copy of *mmoX* which occurs on its own in the chromosome and is separate from the usual *mmoXYBZDC* cluster showed that this second copy is not functional.

During growth of methanotrophs that contain both pMMO and sMMO under conditions where there is a low copper-to-biomass ratio, transcription of *mmo*R and *mmo*G and correct folding of MmoR may occur. The latter may then form a complex with RNA polymerase containing σ^{N} which facilitates transcription of *mmo*XYBZDC. Alternatively, during growth in medium where there is a high copper-to-biomass ratio, MmoR is inactivated directly or via MmoG by an as yet unknown mechanism. Two further genes, *mmoQ* and *mmoS*, which are homologous to two-component signaling systems in other bacteria, are found adjacent to the structural and regulatory genes in *Mc. capsulatus* (Bath) and could be involved in copper sensing. However, the exact mechanisms by which copper interacts directly (or indirectly) with MmoR to prevent transcription, or how the cells sense the

intracellular or extracellular levels of copper which switch of expression of sMMO, are unclear (reviewed in Hakemian and Rosenzweig 2007).

Expression of the *pmo*CAB cluster during growth on medium containing excess copper ions occurs via a σ^{70} activated promoter located 5′ of *pmo*C. In the absence of copper ions, pMMO genes are still expressed, albeit at lower levels, but the apoenzyme produced is inactive. This inactive pMMO can be activated in vitro by the addition of copper ions. Again the exact mechanism by which pMMO is regulated is not known. Interestingly in *Methylocella silvestris*, which does not contain pMMO, the expression of soluble MMO is not repressed by copper ions but instead is repressed by the presence of multicarbon substrates such as acetate (Crombie and Murrell 2014). Methanobactin, a copper-chelating siderophore-like molecule of 1,217 Da, binds copper with high affinity. Methanobactin was first isolated from spent medium of *Ms. trichosporium* and *Mc. capsulatus* grown at low copper, and the metal-binding properties of this chalkophore have been studied in some detail (e.g., see Choi et al. 2005, 2006; DiSpirito et al. 1998; Kim et al. 2004, 2005). Its crystal structure has also been elucidated. Methanobactin is probably involved in copper uptake and may also play a role in pMMO activity (reviewed in Balasubramanian and Rosenzweig 2008). Recent evidence suggests a model for the copper switch mechanism and regulation of the sMMO operon and pMMO operon which involve methanobactin and MmoD. At low copper ion concentration, MmoD acts to repress the pMMO operon and also upregulates expression of the *mbn* operon to produce methanobactin. Methanobactin in turn increases the expression of the *mmo* (sMMO-encoding) operon which further represses pMMO expression. In the presence of excess copper ions, methanobactin is bound to copper and is unable to upregulate sMMO expression. The MmoD protein also binds copper and is unable to repress pMMO (Semrau et al. 2013, 2018; DiSpirito et al. 2016). The identification of a constitutively sMMO-expressing mutant of *Ms. trichosporium* with a deletion of part of the copD gene led to the suggestion that the *copCD* system is involved in copper regulation in methanotrophs (Kenney et al. 2016). The *copCD* genes encode for a copper-binding protein and inner membrane protein, respectively, and are utilized by other bacteria for copper uptake. However specific knockout mutants of *copCD* in *M. trichosporium* OB3b suggest this is not the case (Gu et al. 2017).

5 Methanotrophs in Biocatalysis and Bioremediation

Interest in methanotrophic bacteria as biocatalysts for synthetic chemistry and bioremediation stems almost exclusively from the unique catalytic properties of the two MMO systems, most importantly their ability (a) to oxidize methane to methanol and (b) to co-oxidize a wide range of other substrates. Both systems require an exogenous source of reductant for the monooxygenation reaction, which in whole-cell applications can be supplied from added methanol or formate, via the principal enzymes of methylotrophic metabolism that are also present in

the cells. In addition, the presence of oxygen-stable hydrogenase activity in methanotrophs enables hydrogen to be used as the reductant.

sMMO can co-oxidize a remarkable range of alkanes, alkenes, cyclic alkanes, aromatic compounds, and substituted aliphatic and aromatic compounds even though methanotrophs cannot grow on these compounds (reviewed in Smith and Dalton 2004; Smith and Murrell 2008). Singly oxygenated products predominate with all substrates. Alkanes are hydroxylated, in the case of aliphatic compounds almost exclusively at the terminal and subterminal positions. Ring hydroxylation of aromatics occurs primarily at the meta position, along with a comparable amount of substituent hydroxylation when an alkyl substituent is present. sMMO oxygenates alkenes to epoxides with retention of stereochemistry around the C = C double bond. Ethers are cleaved oxidatively to yield mixtures of alcohols and aldehydes, and pyridine undergoes N-oxygenation. The initial oxygenated products formed from halogenated substrates may decompose rapidly via nonenzymatic pathways that result in the loss of halogen substituents. It is certain that there are many substrates of sMMO that have simply never been tested with the enzyme. A very few small organic compounds are known not to be effective substrates of sMMO. These include tetrachloromethane, iodomethane, trimethylamine, and tetrachloroethene (reviewed in Smith and Dalton 2004; Smith and Murrell 2008).

An extensive study was performed in the 1990s by Dalton and co-workers toward developing sMMO-expressing *Mc. capsulatus* cells for production of epoxypropane from propane. In this pilot process, methanol was used as the reductant, and inhibition of sMMO by the epoxide product was overcome by operating the process in a continuous two-stage system that allowed epoxide-inhibited culture to recover in a separate bioreactor in the presence of methane and other nutrients. The process gave good productivity and had the advantage that at 45 °C (the optimal growth temperature of *Mc. capsulatus*) the epoxypropane product was easily recovered from the gas phase. With cells at 30 g L^{-1}, the epoxypropane production rate was 250 g L^{-1} day^{-1}, and the total cost of epoxypropane production was estimated at US$1.26 per kg (Richards et al. 1994). The process came close to reaching the same cost as the established commercial chemical technology but did not offer a financial advantage over the existing technology so has not yet been commercialized, although patents for the process were filed worldwide. The process was also evaluated for production of 1,2-epoxybutane from but-1-ene and acetaldehyde from ethane.

sMMO and pMMO are attractive biocatalysts for conversion of methane to the liquid fuel methanol, which is fuel with a higher energy density than methane that is also easier to store and transport (Bjorck et al. 2018). Development of a suitable cell-free MMO system or cells engineered to minimize onward metabolism of methanol may enable such technology.

Recently a site-directed mutagenesis study of MmoX has identified a mutant R98L that abolishes a salt bridge on the periphery of the hydroxylase (Fig. 4). This mutant has increased activity toward aromatic substrates and altered regioselectivity for more precise hydroxylation of the substrate biphenyl (Lock et al. 2017). This unique enzyme co-oxidizes a wide range of organic substrates, and it will be

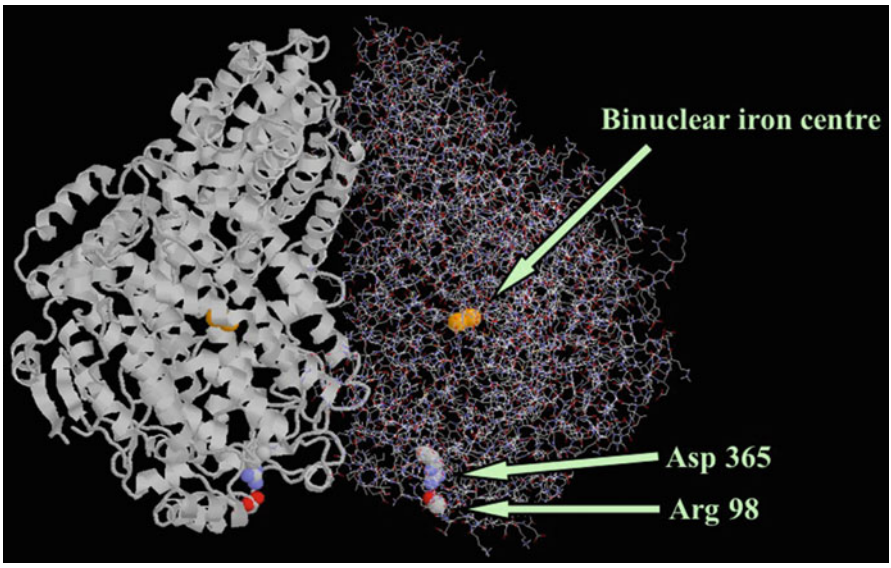

Fig. 4 Structure of the hydroxylase component of sMMO from *Ms. trichosporium* OB3b showing the position of the mutated residue Arg 98 and Asp 365, with which it forms an ionic interaction

interesting to ascertain if its properties can be further enhanced by either random or directed mutagenesis and gene shuffling, for example, to co-oxidize polyaromatic hydrocarbons or make chiral epoxides or alcohols, thus improving its biotechnological potential even further.

The diverse co-oxidation reactions catalyzed by sMMO and pMMO have led to many suggested applications in the oxidation of environmental pollutants (reviewed in Smith and Dalton 2004). The priority pollutant trichloroethylene (TCE) is a substrate for both forms of MMO (see Lee et al. 2006), and by a combination of enzyme-catalyzed oxygenation and nonenzymatic steps, pMMO-expressing methanotroph cells can lead to its mineralization to CO_2, water, and chloride. There has been a large number of pilot studies into the use of methane-oxidizing bacteria for bioremediation of groundwater and effluents contaminated with TCE and other chlorinated solvents. During a long-term study, a TCE-contaminated aquifer in Japan has been periodically biostimulated with methane and inorganic nutrients to promote growth of methanotrophic bacteria to degrade the TCE. Here a stable and significant (10%) decrease from the initial concentration of TCE (200 ppb) was observed from 40 days after beginning biostimulation with methane. The TCE concentration returned to its initial level after biostimulation ceased. Pilot ex situ systems for bioremediation of chlorinated organic solvents using methanotrophs have included practical and financial evaluation of a two-stage process where a mixed methanotroph culture was employed at low copper-to-biomass ratio (to promote sMMO expression) in order to purify effluent contaminated with TCE and *cis*-1,2-dichloroethylene (cDCE). Here, up to 99% removal of

TCE or cDCE (initial concentration 2.25 mg L^{-1}) was achieved. Competition between methane and the chlorinated co-substrate for the (s)MMO active site was avoided by growing the cells on methane in the growth reactor and then mixing with the contaminated wastewater in the second-stage reactor (a plug flow reactor), where formate was added in the absence of methane to supply the reducing equivalents required by MMO (see reviews by Smith and Dalton 2004; Smith and Murrell 2008 and references therein for further detail on bioremediation and biocatalysis by methanotrophs). By utilizing gene probe hybridization, it was suggested that the majority of TCE biodegradation at a field test site in Carolina, USA, was carried out by sMMO-expressing bacteria (Hazen et al. 2009). It has been shown that a facultative methanotroph *Methylocystis* strain SB2 constitutively expresses pMMO when grown on multicarbon substrates and is able to degrade a variety of chlorinated hydrocarbons including TCE, *trans*-dichloroethylene, vinyl chloride, and 1,1,1-trichloroethane (Im and Semrau 2011; Yoon et al. 2011). Increasing understanding of the way methanotrophs expressing pMMO and sMMO interact with other microorganisms in complex communities is expected to lead to further exploitation of cells expressing these enzymes in bioremediation and other biotechnologies.

Other possibilities for bioremediation using methanotrophs include use of sMMO-expressing cells to facilitate biodegradation of mono- and di-aromatic pollutants (including polychlorinated biphenyls) by introducing oxygen functionality into these recalcitrant molecules. In the longer term, methanotrophs expressing recombinant sMMO enzymes with increased substrate range or regioselectivity may be developed for novel biotechnological applications using the mutagenesis system mentioned earlier (reviewed in Smith and Murrell 2008).

6 Research Needs

There are a still a number of challenges in the study of methane monooxygenases and their regulation. Recently an expression system for pMMO has been developed within *Escherichia coli*, which is capable of producing active PmoB, capable of methane oxidation (Balasubramanian et al. 2010). This has allowed further insights into the nature of the active site and enables future site-directed mutagenesis studies to elucidate the exact catalytic mechanism. The exact nature and function of the copper centers in pMMO still need to be further defined. Also the in vivo electron donor and pathways of electron transfer to pMMO are not yet known. It will also be interesting to learn the exact function of methanobactin in MMO regulation, copper sequestration, and delivery of copper ions to the active site of pMMO which is still unclear (DiSpirito et al. 2016; Kenney and Rosenzweig 2018). The availability of the genome sequence of *Mc. capsulatus*, together with a facile genetic system, will facilitate the study of copper transport/uptake systems in methanotrophs and help determine exactly how copper regulates the expression of sMMO and pMMO in methanotrophs that contain both enzyme systems. The role of two-component systems in methanotrophs with respect to regulation of methane oxidation also needs attention as does the mechanism of regulation of sMMO by multicarbon

compounds in *Methylocella silvestris*. A good system for the expression and mutagenesis of sMMO from *Ms. trichosporium* is now available. This will enable researchers to define the exact nature of the active site of sMMO and what makes this enzyme unique in being able to oxidize methane and also enable mutation of sMMO in order to alter its catalytic utility. The ability of methanotrophs to produce valuable bioproducts using methane as a feedstock is another focus for biotechnological research. It has been shown that methanotrophic bacteria can produce a variety of valuable products such as liquid biofuel, polyhydroxyalkanoate bioplastics, single-cell protein for animal feed, and other bioproducts such as ectoine and vitamin B12 (Strong et al. 2015; Pieja et al. 2017; Cantera et al. 2018), and this is showing great potential for future development of MMO and methanotrophs as a commercially viable biotechnology.

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