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Methane-Oxidizing Enzymes: An Upstream Problem in Biological Gas-to-Liquids Conversion

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ABSTRACT: Biological conversion of natural gas to liquids (Bio-GTL) represents an immense economic opportunity. In nature, aerobic methanotrophic bacteria and anaerobic archaea are able to selectively oxidize methane using methane monooxygenase (MMO) and methyl coenzyme M reductase (MCR) enzymes. Although significant progress has been made toward genetically manipulating these organisms for biotechnological applications, the enzymes themselves are slow, complex, and not recombinantly tractable in traditional industrial hosts. With turnover numbers of 0.16−13 s^{-1}, these enzymes pose a considerable upstream problem in the biological production of fuels or chemicals from methane. Methane oxidation enzymes will need to be engineered to be faster to enable high volumetric productivities; however, efforts to do so and to engineer simpler enzymes have been minimally successful. Moreover, known methane-oxidizing enzymes have different expression levels, carbon and energy efficiencies, require auxiliary systems for biosynthesis and function, and vary considerably in terms of complexity and reductant requirements. The pros and cons of using each methane-oxidizing enzyme for Bio-GTL are considered in detail. The future for these enzymes is bright, but a renewed focus on studying them will be critical to the successful development of biological processes that utilize methane as a feedstock.

INTRODUCTION

Methanotrophs, aerobic organisms that utilize methane as a carbon and energy source, were first discovered in 1906.[1−4] Their unique metabolic lifestyle is enabled by metalloenzymes known as methane monooxygenases (MMOs), which catalyze the first step in the methanotroph metabolic pathway, the oxidation of methane to methanol.[5−8] Methanol is further oxidized to formaldehyde and formate, which are either assimilated for biomass production or dissimilated to CO_2 for energy production, thus forming an oxidative arm of the global carbon cycle. Methanotrophs can utilize the serine cycle similar to methylotrophs, organisms that metabolize methanol, or they can use the ribulose monophosphate cycle (RuMP) for carbon assimilation.[9−11] Indeed, much of our understanding of microbial one-carbon (C1) assimilation pathways derives from a mix of results obtained with methanotrophs and methylotrophs, and the history of these organisms is intertwined.[1] In this context, methanotrophs can be thought of as methylotrophs endowed with MMOs. While this is an oversimplification of methanotrophy since methane oxidizers have evolved a number of biochemical systems specific to methane,[2,3,12,13] it highlights the importance of MMOs in harnessing the biotechnological value of methane.

MMOs have been chiefly of interest to bioinorganic chemists since their discovery 66 years ago,[5,7,14] but the recent availability of inexpensive natural gas has sparked intense interest from the biotechnology community in these enzymes and the organisms that produce them.[15−17] In particular, MMOs have the potential to enable the use of methane as a carbon feedstock for industrial biochemical processes instead of high-cost sugars, which are estimated to be 50% of the cost of production of the final fuel or chemical.[17] Preliminary analysis suggests biological gas-to-liquids (Bio-GTL) technology, driven mainly by lower capital expenditures, can be competitive with Fischer−Tropsch GTL on small scales (<10 000 barrels per day) if energy and carbon efficiencies similar to ethanol fermentation can be achieved.[15,16] Critically, high volumetric productivity, which is a function of the methane oxidation and mass transfer rates, carbon conversion efficiency, and catalyst/cell density, is needed. Best and worst-case analysis indicates that the cost of raw materials from methane-derived diesel based on a methanotroph process could range from $0.7/gal to $10.8/gal.[17] At the time of this analysis (2014), the cost of raw materials for diesel production derived from crude oil was $2.3/gal.[17] Using butanol as a final product, techno-economic analysis indicates that Bio-GTL could have been economically viable in the context of oil and gas prices at 2014 levels if state-of-the-art technology had been available and scalable.[15,16] A number of technologies that would make Bio-GTL economical with crude oil prices in the $50−60 per barrel range and natural gas prices below $4 per mmBTU were also proposed.[16] Although such analyses are very preliminary, they suggest that methanotroph-derived fuels have the potential to compete in the conventional market.

Selective activation of the methane C−H bond (105 kcal/mol)[18] is the key challenge in GTL processes. MMOs are the ideal catalysts in this regard because they selectively oxidize methane to methanol at ambient temperature and pressure.[5−7,19] However, there are many questions related to the practicality of using methanotrophs and/or their enzymes for high-volume commercial production of low-value products like fuels or commodity chemicals.[15−17,20] For example, are MMOs fast enough to achieve economical volumetric productivities (i.e., the upstream problem)? Are they robust enough to resist inactivation by common contaminants found in natural gas? Can sufficient energy and carbon efficiencies using aerobic C−C bond forming pathways be obtained? Can industrial strains of methanotrophs be engineered, or can MMOs be expressed in

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industrially relevant host organisms? If not MMOs, are there viable alternatives? One possibility is to exploit the anaerobic oxidation of methane (AOM) performed by archaea (ANME) growing in consortia with bacteria that reduce inorganic compounds.[21−24] Whereas numerous aerobic methanotrophs have been cultured in the laboratory and there is relatively little controversy over the enzymes and the metabolic intermediates involved in aerobic methane oxidation,[1,2,9,10,25] the inability to obtain pure cultures of ANME has complicated the elucidation of its biochemistry, leaving many aspects of AOM still contested.[24,26−30] Ultimately, we must determine whether the methane oxidation enzymes involved in these processes possess commercial potential or are simply toys to be played with in the laboratory.

In the past three years, the answers to some of these questions have begun to unfold, largely due to the Advanced Research Projects Agency - Energy (ARPA-E) Reducing Emissions using Methanotrophic Organisms for Transportation Energy (REMOTE) program. New genetic tools for engineering methanotrophs have been developed,[11,31] and robust strains of methanotrophs have been discovered and characterized.[32−34] Methanotrophs have been engineered to produce butanol, lactate, and carotenoids,[20,35−38] catalytically active MMO-containing polymers have been developed,[39] and novel enzymes and new biochemical pathways for forming C−C bonds from methanol and formate have been designed.[40,41] In addition, genes for methylotrophy have been successfully incorporated into *Escherichia coli*,[42] and strains of methane producing archaea known as methanogens have been engineered to catalyze AOM, thus reversing methane synthesis.[43] Methane mass transfer and bioreactor design have continued to receive attention as well.[44−48] Despite all of this progress, many of the questions regarding the first step of biological methane oxidation remain unanswered. Since the rate of methane oxidation and uptake is a key driver of volumetric productivity, these questions must be explored or the field will be at risk of simply being left with really nice strains of organisms that grow on methanol. As rapper Puff Daddy might say, "It's all about the [methane-oxidizing enzymes], baby." In this Perspective, we summarize the current state of biological methane oxidation with an emphasis on key aspects relevant to biotechnological applications.

AEROBIC METHANE OXIDATION

Interplay between Two Methane Oxidation Enzymes. Methanotrophs oxidize methane to CO_2 in four distinct steps via methanol, formaldehyde, and formate.[9,10] The oxidation of methane to methanol is the only energy-dependent reaction in the methanotroph pathway and can be catalyzed by either particulate methane monooxygenase (pMMO) or soluble methane monooxygenase (sMMO).[1,7,8] pMMO is a 300 kDa integral membrane enzyme that contains a copper active site and is located within extensive intracytoplasmic membranes (ICMs).[49−52] sMMO is a 250 kDa cytoplasmic enzyme that contains a diiron active site.[53,54] Both enzymes require two reducing equivalents and catalyze methane oxidation following the same reaction stoichiometry (Scheme 1). pMMO is nature's predominant aerobic methane oxidation catalyst and is found in almost all methanotrophs. A subset of methanotrophs express both sMMO and pMMO, whereas a small number of organisms contain only the genes for sMMO.[3,9,10] In organisms with both sMMO and pMMO, the regulation of sMMO is tightly controlled by a mechanism referred to as the "copper switch" in which sMMO is expressed under low copper conditions and is dramatically down-regulated in the presence of copper.[52,55−58] In some methanotrophs, the production of a small peptidic copper chelator called methanobactin is co-regulated with sMMO resulting in its secretion under low copper conditions.[3,13,58−60] In methanotrophs that undergo the copper switch, pMMO is slightly upregulated in the presence of copper and is the predominant MMO at high copper concentrations, accounting for up to 20% of the total protein content.[56,58,61,62] Additionally, the presence of ICMs is strongly correlated with pMMO expression and copper availability.[57,61] The mechanism by which these proteins are transcriptionally regulated remains largely unknown. Once methane is oxidized to methanol, it is further oxidized to formaldehyde, which can be assimilated or subsequently oxidized to CO_2 for energy production. Progress on characterizing these downstream steps has been reported elsewhere recently.[10,25,34,63]

Soluble Methane Monooxygenase (sMMO). sMMO requires three proteins for activity: the hydroxylase (MMOH), the regulatory component (MMOB), and the reductase (MMOR).[7,8] Numerous structural studies on sMMO have been performed, and the structures of all three components are known.[53,64−67] Notably, the crystal structure of MMOH has been solved to a resolution of 1.7 Å, in complex with MMOB, in different oxidation states, and in the presence of substrate and product analogues.[68−71] Likewise, over the past 30 years, the kinetics and spectroscopy of sMMO have been studied extensively by multiple investigators,[5,8,72] leading to the emergence of a relatively complete mechanism. Although aspects of this mechanism are still controversial, more than enough information is available to predict how sMMO will behave in Bio-GTL applications.

Genetic, structural, and mechanistic aspects of sMMO have been reviewed recently.[7,8,73] In brief, the genes for sMMO are present in an operon containing 11 genes of which five encode the structural components of sMMO.[74,75] The *mmoXYZ* genes encode the α, β, and γ subunits of the hydroxylase (MMOH), which form an $(\alpha\beta\gamma)_2$ dimer (Figure 1A,B).[19] The site of oxygen activation and methane oxidation is a non-heme diiron center coordinated by two histidines, four glutamates, and two or three water molecules located within a four-helix bundle of the α-subunit (Figure 1B,C).[5] *mmoB* encodes the regulatory component (MMOB), which binds to MMOH (Figure 1A,B) and increases the reaction rate of reduced MMOH with oxygen 1000-fold and the steady-state rate up to 150-fold.[76,77] Binding of MMOB also decreases the reduction potential of MMOH,[78] affects the structure of the diiron site,[69] and alters accessibility to the active site.[69,77,79,80] *mmoC* encodes the reductase component (MMOR), which contains a [2Fe-2S] cluster in its N-terminal ferredoxin domain and FAD in its C-terminal domain (Figure 1D,E).[19,81] NADH binds in the C-terminal domain and transfers two electrons sequentially to MMOH via FAD and the [2Fe-2S] cluster.[82,83] sMMO turnover is a highly choreographed event in which MMOR and MMOB bind to MMOH, perhaps at the same site, in order to deliver electrons,

Scheme 1. Aerobic Methane Oxidation Catalyzed by pMMO and sMMO

$$CH_4 + O_2 + 2e^- + 2H^+ \xrightarrow{MMO} CH_3OH + H_2O$$

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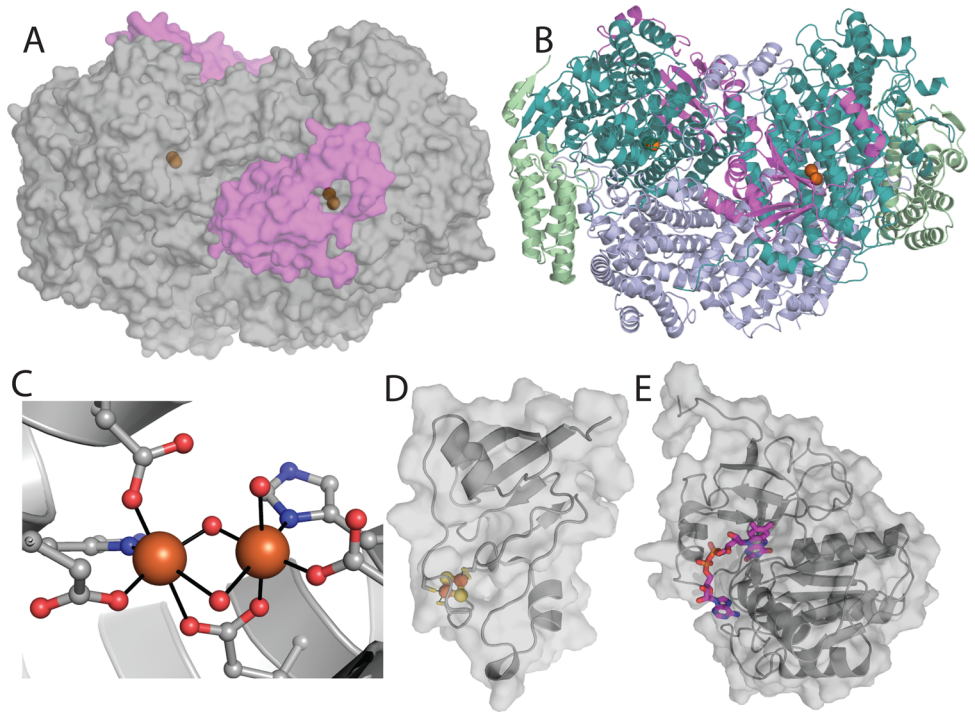

Figure 1. Structures of sMMO from *Methylococcus capsulatus* (Bath). Iron ions are shown as orange spheres. (A) Crystal structure of the MMOH-MMOB complex shown as a surface with MMOH in gray and MMOB in magenta (PDB code 4GAM). (B) Crystal structure of the MMOH-MMOB complex shown as a cartoon with the α subunit in teal, the β subunit in light blue, the γ subunit in light green, and MMOB in magenta. (C) The MMOH diiron site in the diferric form with water and hydroxide ligands shown as red spheres (PDB code 1MTY). (D) NMR structure of the MMOR ferredoxin domain with the [2Fe-2S] cluster shown as spheres (PDB code 1JQ4). (E) NMR structure of the MMOR flavin-binding domain with the flavin shown as magenta sticks (PDB code 1CKV).

induce structural changes at the diiron center, and gate substrate and proton access to the active site.[5,7,8,73]

Reductive activation of O_2 at the sMMO diiron site has been of great interest to bioinorganic chemists, and the key intermediates in this process have been characterized (Figure 2). These include the peroxo intermediates, P* and P, formed from the reaction of O_2 with the reduced MMOH-MMOB complex and the Q intermediates formed from P after cleavage of the O−O bond.[5,7,8,72] In the catalytic cycle, the diiron(III) center is reduced to the diiron(II) form ($MMOH_{red}$) by MMOR and then reacts with oxygen in the presence of MMOB to form intermediate O.[76] This intermediate is converted to the first and second peroxo intermediates, P* and P. The structures of P and P* remain a subject of discussion.[7,8,84,85] Intermediate P can react with substrates like alkenes, ethers, nitriles, nitro alkanes, and aldehydes, but not alkanes,[86−88] and can be generated in vitro without oxygen, electrons, MMOB, and MMOH by reacting the oxidized resting state of MMOH ($MMOH_{ox}$) with peroxide in a reaction commonly referred to as the peroxide shunt.[89,90] Intermediate P undergoes homolytic O−O bond cleavage and proton transfer to form Q, which is believed to be a diamond di(μ-oxo) diferryl core (Figure 2).[84,91,92] Q then reacts with substrate to form the product complex intermediate, T.[8] Alternatively, Q can decay via Q* to $MMOH_{ox}$ in the absence of substrate.[84] The details of C−H activation by Q have been probed by the use of radical clock substrates and chiral alkanes as well as determination of kinetic isotope effects and extensive calculations. Radical, non-radical, and non-synchronous concerted mechanisms have been proposed on the basis of these studies.[8,93−95]

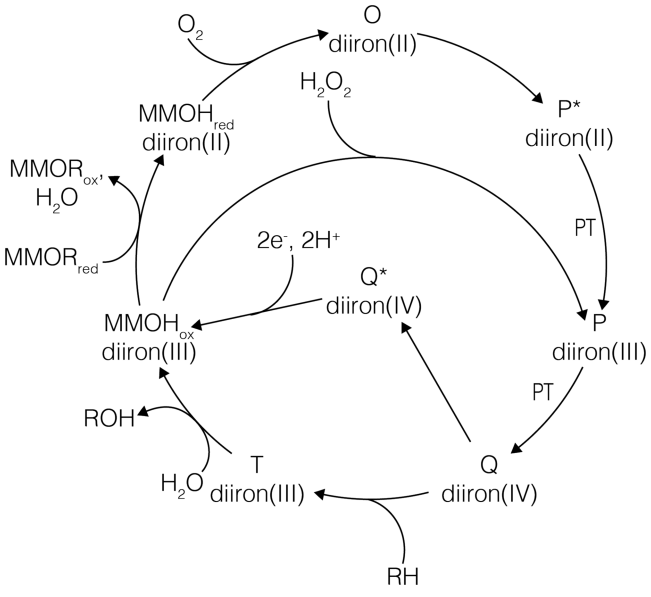

Figure 2. Catalytic cycle of sMMO. PT indicates proton transfer, RH indicates substrate, and ROH indicates hydroxylated product.

Particulate Methane Monooxygenase (pMMO). pMMO is an $(\alpha\beta\gamma)_3$ homotrimer encoded by the genes *pmoCAB* (Figure 3A).[6] The individual components of each subunit are referred to by their gene name. Crystal structures of pMMO from four organisms reveal that all but one also contain an additional helix (helix X) (Table 1).[50,96−98] PmoA and PmoC are integral membrane proteins and do not have extensive soluble domains. In contrast, PmoB includes two

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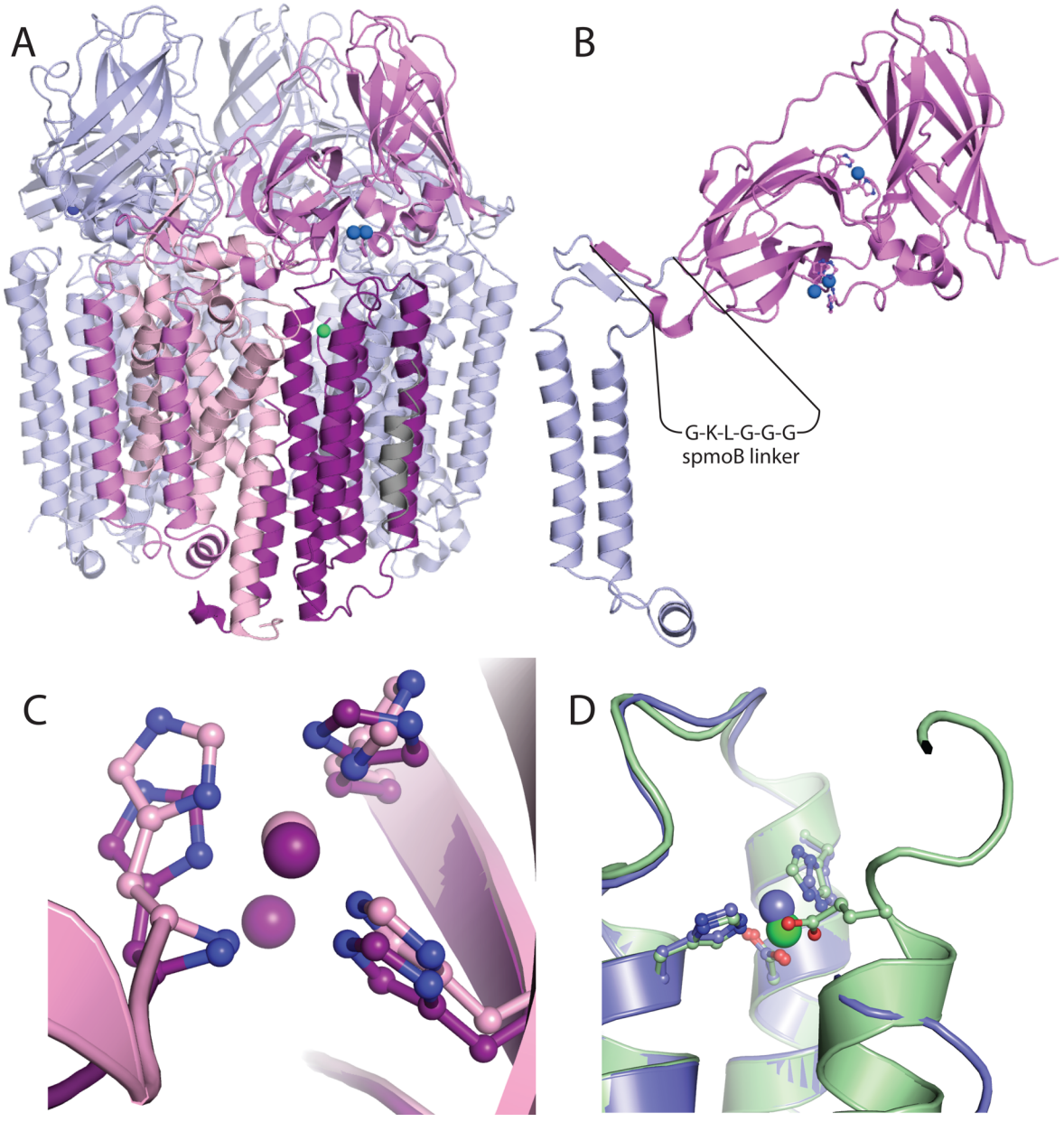

Figure 3. Crystal structures of pMMO. (A) Crystal structure of pMMO from *Methylocystis* species strain M with one protomer colored by subunit and the other two protomers in light blue (PDB code 3RFR). PmoB is shown in violet with the copper ions shown as blue spheres, and PmoC is shown in purple with the zinc ion shown as a green sphere. PmoA is shown in light pink. Helix X is shown in gray. (B) PmoB from *Methylococcus capsulatus* (Bath) with the copper ions shown as blue spheres. The region of PmoB excised to create spmoB is shown in light blue (PDB code 3RGB). The sequence of the spmoB linker is shown as one-letter amino acid codes. (C) Superposition of PmoB subunits from structures of *Methylocystis* species strain M pMMO containing a monocopper center (pink, PDB code 3RFR) and *Methylococcus capsulatus* (Bath) pMMO containing a dicopper center (purple, PDB code 3RGB). (D) Superposition of the PmoC subunits from structures of *Methylocystis* species strain Rockwell pMMO crystals soaked in zinc (green, PDB code 4PI2) and copper (blue, PDB code 4PI0).

Table 1. Summary of pMMO Crystal Structures

organism	metals modeled in PmoB N-term site	presence of PmoB monocopper site	metals modeled in PmoC site	helix X	resolution (Å)	PDB code
Methylococcus capsulatus (Bath)	dicopper	yes	Zn	no	2.8	3RGB
Methylosinus trichosporium OB3b	dicopper	no	Cu	yes	3.9	3CHX
Methylocystis species strain M	both	no	Zn	yes	2.6	3RFR
Methylocystis species strain Rockwell	monocopper	no	Cu	yes	2.5	4PHZ
Methylocystis species strain Rockwell + Zn(II)	monocopper	no	Zn	yes	3.1	4PI0
Methylocystis species strain Rockwell + Cu(II)	monocopper	no	Cu	yes	3.3	4PI2

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Table 2. Overview of Biological Methane-Oxidizing Systems

	sMMO	pMMO	spmoB	CYP153A6	ANME MCR
Enzyme Characteristics					
reductant/oxidant	NADH	quinone	quinone	iodosyl-benzene	CoM-S-S-CoB
product	methanol	methanol	methanol	methanol	methyl-CoM
structural proteins	MmoXYZ	PmoCAB	PmoB[a]	CYP153A6	McrABG
oligomeric state	$(\alpha\beta\gamma)_2$	$(\alpha\beta\gamma)_3$	unknown	monomer	$(\alpha\beta\gamma)_2$
molecular weight (kDa)[b]	251	300	32[c]	48	280
additional protein components	MMOR, MMOB	unknown	n/a	n/a	unknown
active site cofactor	iron	copper	copper	heme	F_{430}
localization	cytoplasm	membrane	cytoplasm[d]	cytoplasm[d]	cytoplasm[200]
Kinetics					
K_m (μM)[e]	92[f]	8.3−62[g]	n/a	n/a	>10 000[h]
turnover frequency (s^{-1})[e]	12.9[i]	0.68−2.5[j]	3.72×10^{-4}[k]	8.3×10^{-5} [l]	0.2[m]
k_{cat}/K_m (s^{-1} M^{-1})[e]	140 000[i,f]	40 000−82 000[g,j]	n/a	n/a	<20[h,m]
specific activity (nmol mg^{-1} min^{-1})[e]	6170[i]	410−1500[j]	0.697[k]	0.104[l]	70[m]
exptl carbon efficiency (%)	37.6[n]	49.4[n]	n/a	n/a	0.25−1.3[o]
enzymatic productivity (nmol mg^{-1} min^{-1})	2320	740−200	n/a	n/a	0.7[p]
methane uptake (mmol $gDCW^{-1}$ h^{-1})[e]	21.8[f]	2.5−9.0[g]	n/a	n/a	0.2[q]
Pathway Efficiency[r]					
carbon efficiency (%)	67[s]	67[s]	n/a	n/a	100[t]
energy efficiency (%)	51[s]	51[s]	n/a	n/a	77[t]
Expression Information					
native source	methanotrophs	methanotrophs	*Methylococcus capsulatus* (Bath)	*Mycobacterium* sp. HXN-1500	ANME
genetically tractable native hosts	yes	yes	n/a	n/a	No
culturable	yes	yes	n/a	n/a	No
heterologous hosts[u]	*Pseudomonas*	*R. erythropolis*	*E. coli*	*E. coli*	*M. acetivorans*
known auxiliary systems	Fe−S cluster formation, copper switch	copper homeostasis, copper switch, membrane formation	n/a	n/a	cofactor synthesis (methyl-CoM, CoB, F_{430}), systems for post-translational modification

[a]Engineered sequence. [b]Molecular weight of the experimentally determined oligomeric state unless otherwise indicated. [c]Molecular weight of the engineered protein. [d]Localization in heterologous host. [e]Values are calculated for whole cells unless specified otherwise. [f]Taken from Oldenhuis et al. (1991).[158] Reported values are for *M. trichosporium* OB3b cells expressing sMMO. Methane uptake rate is V_{max} value determined using Michaelis−Menten methods. [g]Taken from Lontoh et al. (1998).[159] Reported values are for *M. trichosporium* OB3b cells expressing pMMO. Methane uptake rate is V_{max} value determined using Michaelis−Menten methods assuming 50% of dry cell weight is protein. [h]Taken from Nauhaus et al. (2007) and Thauer (2008).[27,140] [i]Calculated from the rate of methane uptake[158] assuming 50% of cells is protein and 11.67% of protein is sMMO.[201] Reported values are for sMMO from *M. trichosporium* OB3b.[158] Reported turnover number is k_{cat} with an error of ∼14%. [j]Calculated from the rate of methane uptake[159] assuming 20% of protein is pMMO.[62] Reported values are for pMMO from *M. trichosporium* OB3b. Reported turnover number is k_{cat} with an error of ∼8−19%. [k]Values are for purified protein taken from Culpepper et al. (2014) and have an error of ∼26%.[105] [l]Values are for purified protein taken from Chen et al. (2012) and have an error of ∼40%.[108] [m]Calculated from the rate of methane uptake[140] assuming 50% w/w of cells is protein and 10% of protein is MCR, which is estimated based on the % MCR present in ANME consortia.[27,154] [n]Values for sMMO and pMMO correspond to low and high copper growths of *M. capsulatus* (Bath) reported by Leak and Dalton (1986).[164] [o]Reported value is % methane carbon incorporated into biomass as a fraction of total methane oxidized.[140,165] [p]Calculated assuming a carbon assimilation rate of 1%. [q]Taken from Nauhaus et al. (2007).[140] [r]Calculated based on butanol production. [s]Taken from Haynes and Gonzalez (2014).[16] [t]Taken from Mueller et al. (2015)[147] [u]Excludes expression of foreign MMOs in methanotrophic hosts.

soluble, periplasmic cupredoxins located at the N and C termini, connected in the middle by two transmembrane helices (Figure 3B). The crystal structures show some variability in metal binding (Table 1). In all structures, one or two copper ions are modeled at the N-terminus of the first PmoB cupredoxin domain (Figure 3C). These copper ions are coordinated by an HXH motif derived from β-strand 6 of the cupredoxin fold as well as the amino terminus and imidazole ring of an N-terminal histidine residue.[50] pMMO from *Methylococcus capsulatus* (Bath) binds an additional copper ion, referred to as the monocopper site, with two histidines near the interface of the PmoB cupredoxin domains.[50] It is not surprising that this monocopper site is absent in other pMMOs since only one of the two observed ligands is conserved.[96−98] In addition, PmoC houses a metal binding site in which two histidines and an aspartate bind a copper or zinc ion (Figure 3D). A fourth glutamate ligand has also been observed.[97] The presence of zinc in this site has been attributed to zinc present in the crystallization condition.[50,96] Moreover, zinc inhibits pMMO, likely by binding in the crystallographic site, leading to the hypothesis that the coordinating residues, which are conserved, could be involved in proton transfer.[97] It is unclear

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if copper binding at this site is functionally important. Samples of pMMOs in which copper is observed in the PmoC site have been prepared in the presence of extra copper, while pMMOs that have not been treated with additional copper require zinc to crystallize.[97,98] It may be that metal binding to the PmoC site is not physiologically relevant, but rather needed to stabilize pMMO for crystallization. It is also worth mentioning that a conserved patch of potential metal binding residues is present in PmoA,[99,100] but metal ions have never been observed in this site by crystallography.[50,96−98]

The identity and location of the pMMO active site have been the subject of much controversy,[101−103] but recent work provides strong evidence that pMMO is a copper-dependent enzyme with the copper catalytic site located at the N-terminus of the PmoB subunit.[49,104,105] Importantly, fragments of PmoB termed spmoB (vide infra) exhibit methane oxidation activity albeit at lower levels than native pMMO.[49,105] In addition, mutations to the copper site located in the N-terminus of spmoB abolish activity and reduce copper binding.[49] Finally, optical spectroscopic data show that spmoB is capable of binding oxygen in a similar fashion to full-length pMMO.[104,105] These data indicate that the copper site at the N-terminus of PmoB is the active site, but the nuclearity of the physiologically relevant species remains unresolved. The original assignment of the dinuclear copper site derives from extended X-ray absorption fine structure (EXAFS) data, which reveal a copper−copper distance of ∼2.5 Å. Two copper ions with this short distance can be modeled in the crystal structures of pMMO from *Methylococcus capsulatus* (Bath), *Methylosinus trichosporium* OB3b, and *Methylocystis* species strain M (Table 1).[50,96,98,106] However, this site is best modeled with a single copper ion in the crystal structures of pMMO from *Methylocystis* species strain Rockwell and of two of the pMMO protomers *Methylocystis* species strain M.[96,97] Since all purified pMMO samples are somewhat heterogeneous in copper content and exhibit varying enzymatic activities,[8] it is difficult to correlate activity with copper stoichiometry. Thus, the possibility of a monocopper active site remains. Further understanding of the active site is essential before the mechanism of pMMO can be investigated in a more systematic fashion.

Engineered Proteins. Considerable effort has been expended searching for and engineering enzymes that are less complex than MMOs and are able to oxidize methane. The first report of an engineered enzyme that oxidizes methane was a result of efforts to identify the location of the pMMO active site.[49] A soluble fragment of the PmoB subunit, spmoB, can convert methane to methanol, and upon mutation of the HXH motif of the N-terminal copper binding site, this activity is abolished.[49] The spmoB protein was generated by replacing the two transmembrane helices of PmoB from *Methylococcus capsulatus* (Bath) with a genetically encoded six amino acid linker and removing the signal peptide, resulting in an ∼32 kDa protein (Figure 3B).[107] This protein expresses as inclusion bodies that can solubilized in urea and refolded in the presence of copper to yield a protein containing ∼2.8 copper ions, consistent with ∼2 copper ions in the N-terminal site and ∼1 copper ion in the monocopper site.[105,107] N-terminal sequencing of this construct reveals that the expressed peptide contains an N-terminal methionine, not the authentic N-terminal histidine, suggesting the N-terminal amino group is not strictly necessary. Turnover rates as high as 0.0033 s^{-1} using duroquinol as a reductant have been reported.[49] This value corresponds to 62% of the activity of full-length pMMO if performed under similar conditions and to 0.14% of the activity of whole cells expressing pMMO (Table 2). Although these initial studies are promising, the spmoB refolding process is highly variable, and typical values of spmoB turnover are generally closer to 3.7×10^{-4} s^{-1} (Table 2).[105] Thus, spmoB has been a valuable tool for studying pMMO, but additional efforts are needed to design variants that express solubly and exhibit improved activity.

Besides native sMMOs, members of the ammonia monooxygenase/pMMO superfamily, and spmoB, the only other enzymes known to oxidize methane are certain cytochrome P450 enzymes (CYP) using non-native chemistry.[108] CYPs belong to a huge family of cysteine-ligated heme enzymes (Figure 4) that catalyze oxidative reactions on a wide variety of endogenous and exogenous substrates and can be expressed readily in *E. coli*.[109−113] Many CYPs are monooxygenases that follow the same reaction stoichiometries as sMMO and pMMO except that other substrates take the place of methane.[114,115] The physiological substrates of CYP monooxygenase range from drugs and other xenobiotics in mammals to alkanes in environmentally relevant microorganisms.[109,110] Similar to sMMO, CYPs also require a reductase component, which typically contains iron−sulfur clusters and/or flavins.[114] The general mechanism for CYP has been reviewed recently.[116−119] In brief, the binding of substrate displaces a water molecule coordinated to the ferric heme, triggering a change in most, but not all, CYPs from low to high spin with a concomitant change in reduction potential thereby priming the enzyme for reduction.[117,120−123] Following one-electron reduction to the ferrous heme, oxygen binds and undergoes an additional one-electron reduction followed by a series of proton transfers

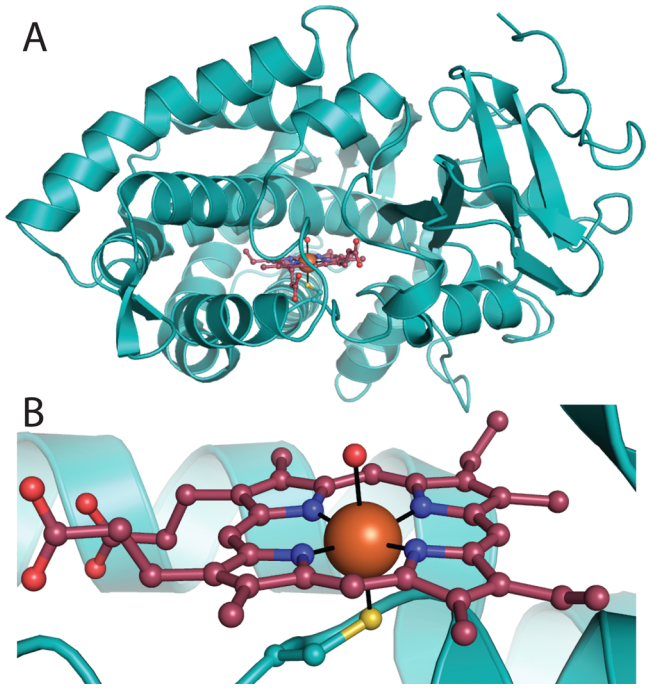

Figure 4. Crystal structure of CAM (PDB code 1PHC). (A) Cartoon representation of CAM without substrate bound. The heme cofactor is shown in red. (B) Zoomed-in view of CAM active site with bound water shown as a red sphere and the sulfur atom of the cysteine ligand shown in yellow.

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resulting in cleavage of the O−O bond to form compound I and a water molecule.[116,117,124] Compound I is a highly reactive Fe(IV)-oxo species,[125] which abstracts a hydrogen atom from the substrate to form a Fe(IV)-hydroxide intermediate referred to as compound II.[118,126] Compound II rapidly recombines with the substrate radical to yield the ferric heme resting state and the hydroxylated product.[118,127] Peroxide shunt and decay pathways similar to those described for sMMO exist as well.[117] Additionally, members of the CYP153 family that are capable of oxidizing C6−C11 alkanes in the terminal position have been identified.[112,128] Given their abundance (more than 20 000 sequences have been identified),[100] their biochemical and catalytic properties, and the advanced state of understanding CYP mechanism, they are a natural starting point for engineering simplified MMOs.

Two strategies have been pursued to create CYP-based systems to oxidize small alkanes using the native catalytic cycle. In the first approach, methods based on rational design and directed evolution have been used to engineer CYPs that can utilize ethane as their smallest substrate. Rational design focused on decreasing the volume of the active site of CYP101 from *Pseudomonas putida* (CAM), which natively oxidizes camphor, was used to engineer an enzyme with nine mutations that oxidizes ethane to ethanol at a rate of 1.32 s^{-1} with 10.5% of NADH oxidation resulting in productive ethane oxidation.[129,130] Notably, the CAM variant with the most ethane oxidation activity contained 45% high-spin heme without substrate bound, suggesting that the spin shift is partially uncoupled from substrate binding.[130] Directed evolution has also been successful in engineering CYPs to oxidize small substrates. CYP102A1 from *Bacillus megaterium* (BM3) was subjected to multiple iterations of library construction and screening in which the size of the substrate was reduced from octane to propane/dimethyl ether and then to ethane.[131] The variant with the highest activity and catalyzing the most turnovers contained 17 mutations and oxidized ethane to ethanol at a rate of 0.0066 s^{-1} with <1% of NADPH oxidation resulting in productive turnover.

The second approach involves using perfluorocarboxylic acid (PFC) "decoy molecules" that trick native CYP into a high-spin state by mimicking substrate binding. The decoy molecule only partially fills the active site so that it is appropriately sized for binding small alkanes and is not hydroxylated by CYP.[132−135] Using this approach, ethane is the smallest hydrocarbon for which activity has been reported. In these studies, numerous decoy molecules have been screened against native BM3. Thus far, $CF_3(CF_2)_8$-L-leucine (PFC9-L-Leu) has the best activity toward ethane, with a rate of 0.066−0.25 s^{-1}.[136] Neither of the two outlined approaches has successfully resulted in methane oxidation driven by NADH. These studies suggest that small substrates by themselves are unable to trigger reduction of the heme iron, which is regulated by the spin-state shift and may be necessary to control through other mechanisms.

Methane oxidation by CYPs has only been achieved by using terminal oxidants, which generate compound I from the ferric heme resting state, thereby eliminating the need for substrate induced effects (Figure 5). Using iodosylbenzene as an oxidant, CYP153A6 from *Mycobacterium* sp. HXN-1500 (A6), which natively oxidizes C6−C11 alkanes, can convert methane to methanol at a rate of 8.3 × 10^{-5} s^{-1}.[108,137] Notably, only A6 and a variant of A6 oxidize methane, whereas BM3, CAM, and a variant of BM3 were unable to oxidize methane in the presence of iodosylbenzene.[108] Additionally, A6 could not oxidize methane using NADH or peroxide, and a spin shift was not observed optically in the presence of 90 mM methane. Collectively, studies with "decoy molecules", variant CYPs, and terminal oxidants suggest that, while the poor binding of small substrates is a barrier to methane activation by CYP, compound I is a powerful enough oxidant to break the methane C−H bond.

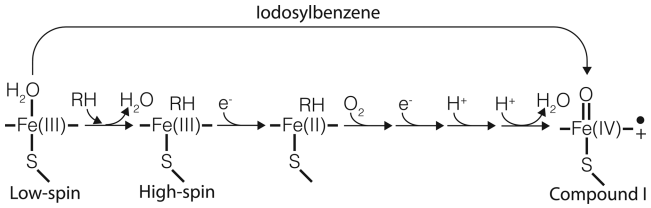

Figure 5. Generation of compound I in cytochrome P450.

ANAEROBIC METHANE OXIDATION AND METHYL-COENZYME M REDUCTASE

Unlike methanotrophs that use oxygen as a terminal electron acceptor, AOM involves consortia of anaerobic methanotrophic archaea (ANME) and bacterial reducers that couple methane oxidation to the reduction of inorganic compounds likely through direct intercellular electron transfer.[24,26,28,29,138,139] In this process, methane is oxidized to CO_2 by ANME with less than 1% of carbon being assimilated.[140] The relationship between ANME and bacterial reducers present in AOM consortia has historically been considered to be syntropic; however, it has been demonstrated that some ANME are capable of reducing sulfate to zerovalent sulfur.[141] AOM has been observed using sulfate, nitrate, iron and manganese as electron acceptors, of which sulfate has the longest history and has been the most heavily investigated.[142−145] The thermodynamic feasibility of these processes has been reviewed in detail.[27,29] These calculations support the plausibility of AOM processes postulated to date, but also indicate that consortia that couple methane oxidation to sulfate reduction grow near thermodynamic equilibrium.

Numerous AOM mechanisms have been proposed with reverse methanogenesis being the prevailing hypothesis.[27,28,146] Direct evidence for this pathway has not been obtained, however. For the purposes of this Perspective, we limit discussion to methyl-coenzyme M reductase (MCR) and homologues found in ANME (ANME MCR) since these enzymes are proposed targets for Bio-GTL processes.[16,147] Methanogens are archaea that convert acetate or oxidized C1 compounds to methane.[148] Using an active site containing the nickel cyclic tetrapyrrole coenzyme F_{430}, MCR catalyzes the final step of methane synthesis in which methyl-coenzyme M (methyl-CoM, mercaptoethanesulfonate) is reductively demethylated using coenzyme B (CoB, mercapto-heptanoyl-threonine phosphate) to form methane and the heterodisulfide CoM-S-S-CoB.[148−150] Using MCR from methanogens, it has been shown that this reaction is reversible.[151] This finding combined with the following evidence has led to the hypothesis that the first step of AOM is catalyzed by an MCR homologue (Scheme 2). First, genes for homologues of MCR have been found in ANME isolates (ANME MCR).[152,153] Second, MCR homologues are present in large concentrations in AOM consortia (~7% ANME group 1, ~3% ANME group 2) and can be purified from these isolates.[29,154,155] Third, methanogens have been engineered to grow on methane by expressing

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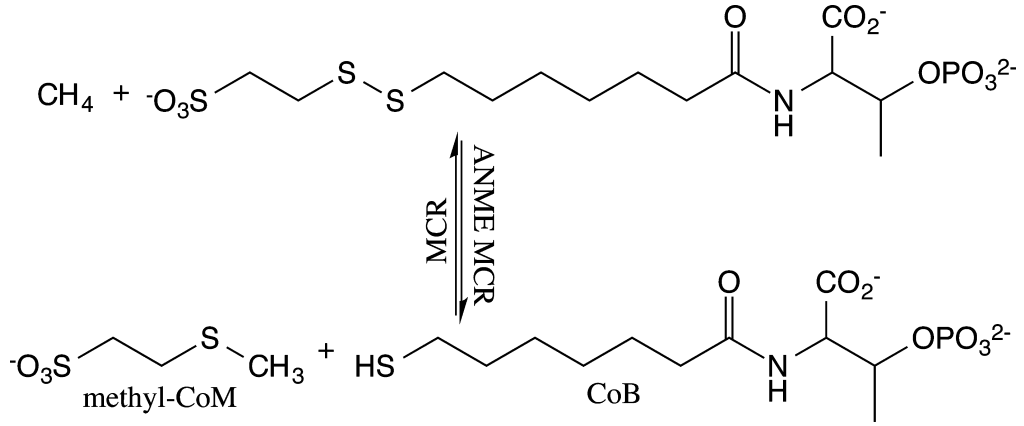
Scheme 2. Anaerobic Methane Oxidation Catalyzed by ANME MCR



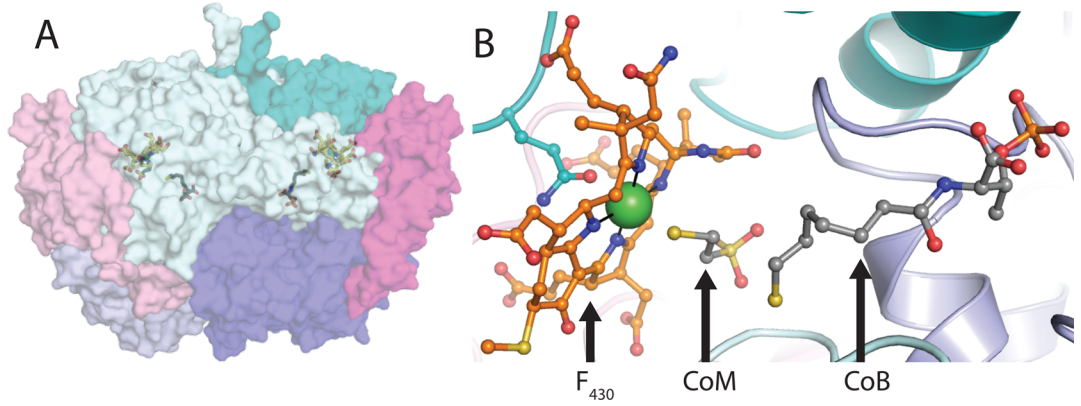


Figure 6. Crystal structure of ANME MCR (PDB code 3SQG). The F_{430} cofactor is shown as orange sticks, and CoM and CoB are shown as gray sticks. (A) The ANME MCR dimer is shown as a surface with the α subunits shown in teal and light cyan, β subunits shown in blue and light blue, and γ subunits shown in magenta and light pink. (B) Zoomed-in view of the ANME MCR cofactors.

ANME MCR.[43] Additionally, the crystal structure of MCR from ANME group 1 has been determined (Figure 6) and differs from the methanogen MCR in changes to the F_{430} cofactor, the presence of a cysteine-rich patch near the F_{430} cofactor, and an altered pattern of post-translational modifications.[156,157] It is unclear which, if any, of these modifications are critical for reversing MCR chemistry or even how well these modifications are conserved across ANME MCR. Despite the many lines of evidence pointing to ANME MCR as the key enzyme for activating methane in AOM, it has yet to be isolated in an active form. Importantly, though, no other enzymes identified from genomic analysis of ANME isolates resemble known methane-oxidizing enzymes.[152,153] MCR thus remains the most tractable target for developing anaerobic methane oxidation biotechnology.

APPLICATIONS IN BIOLOGICAL GAS-TO-LIQUIDS CONVERSION

Comparison of Kinetics, Efficiency, and Atom Economy of Methane Oxidation Enzymes. Biocatalysts for methane oxidation are slow, with turnovers ranging from 0.2 to 12.9 s^{-1} *in vivo* (calculated from experimentally determined methane uptake and protein concentration, Table 2).[158,159] For comparison, the median k_{cat} of enzymes in central metabolism is 79 s^{-1}.[160] Likewise, the catalytic efficiencies (k_{cat}/K_m) of methane-oxidizing enzymes are lower than the median value of enzymes involved in central metabolism, which is 410 000 s^{-1} M^{-1} (Table 2).[160] While the poor kinetics of methane oxidation would seem limiting, the maximum rates of methane uptake under methane saturating conditions for cells producing pMMO and sMMO are 9 and 21.8 mmol $gDCW^{-1}$ h^{-1}, respectively (Table 2).[158,159] For comparison, glucose uptake in *E. coli* is typically <10 mmol glucose $gDCW^{-1}$ h^{-1} or <60 mmol carbon $gDCW^{-1}$ h^{-1} on an atom basis.[161,162] Thus, methanotrophs can overcome slow rates by highly expressing MMOs, resulting in carbon uptake rates that are comparable to sugar-based metabolism (Table 2). However, ANME methane uptake rates are substantially lower than glucose uptake rates. Considering the high levels of MMO expression, methanotrophs grown at modest densities are potentially limited by mass transfer based on experimentally determined mass transfer coefficients for methane, which are highly dependent on reactor design.[44−46] Similar challenges exist for oxygen since the Henry's law constants for methane and oxygen are similar [$(0.92–1.5) \times 10^{-3}$ and $(1.2–1.3) \times 10^{-3}$ M ATM^{-1}, respectively], with the added complication that both gases are needed for aerobic methane oxidation.[163]

In general, the cost of making biosynthetic machinery is not considered since it is assumed biocatalysts will remain active for long periods of time under growth limiting conditions. It is unclear whether this assumption holds true for methane oxidation because the lifetime of these enzymes is unknown. The problem is potentially compounded by the quantity of enzyme needed to support high methane uptake rates, the size of the enzymes, the specialized auxiliary systems required to

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assemble and sustain active methane-oxidizing enzymes, and the slow rate of methane oxidation (Table 2). For example, pMMO from *M. capsulatus* (Bath) contains 5013 carbon atoms, all of which are derived from methane. At a turnover of 2.5 s^{-1}, it would take about 34 min for pMMO to fix enough carbon to create another copy of pMMO. Likewise, the cost of producing ICMs and maintaining copper homeostasis, which are auxiliary systems specific to pMMO, should be considered. Given that methane-oxidizing enzymes differ in terms of the required auxiliary systems and redox couples, it is useful to devise a metric for comparing them that includes the sum total cost of all enzymes and machinery in the cell for their biosynthesis. A simple way to do this is to multiply the specific activity of the methane-oxidizing enzyme (nmol min^{-1} mg^{-1}) by the experimentally determined C_{eff} (carbon utilized for biomass plus excreted products per methane consumed) for the organism (herein referred to as enzymatic productivity). The resulting value is then the maximum rate of carbon assimilation from methane normalized by the quantity of MMO or MCR required to achieve the rate. This approach is useful for comparing catalysts that use their substrate and product for their own synthesis because it accounts for CO_2 lost as a result of energy production and biosynthesis. This metric should only be used to compare enzymes, and broader interpretations of its meaning should be avoided. Based on this analysis, sMMO is only 3 times more productive than pMMO despite being 5 times faster. The difference between MMOs and ANME MCR is much more significant with ANME MCR being 1−2 orders of magnitude slower than MMO, with an enzymatic productivity 3−4 orders of magnitude worse than MMOs (Table 2). This analysis also shows that the rates of spmoB and engineered CYPs need to be improved by several orders of magnitude to be comparable to native MMOs.

Beyond specific activity, proposed MMO-based and MCR-based pathways to fuel differ in their carbon efficiencies (C_{eff}) and energy efficiencies (E_{eff}) (Table 2). In this context, C_{eff} is defined as the molar quantities of carbon in the product divided by the total methane consumed by the pathway. C_{eff} for butanol production from sugar is calculated to be 67%, while proposed pathways for MMO-based and MCR-based butanol production are calculated to have C_{eff} values of 67% and 100%, respectively.[16,147] E_{eff} is defined as the lower heating value (LHV) of the products divided by the LHV value of the methane used in the pathway. E_{eff} for butanol production from sugar is 67%, while proposed pathways for MMO-based and MCR-based processes are calculated to have E_{eff} values of 51% and 76.5%, respectively.[16,147] These calculations highlight a fundamental limitation to methanotrophic assimilation pathways. Specifically, a third of carbon is lost in the assimilatory pathways as CO_2, and the requirement for electrons to catalyze MMO-based chemistry results in lower energy efficiencies.[16] By contrast, MCR-based pathways do not have these limitations,[147] making a strong case that such assimilation pathways will outperform aerobic methanotrophy.

However, calculating C_{eff} and E_{eff} for fuel producing pathways does not take into account the free energy change for the entire metabolic process, which ultimately determines how much methane is assimilated into biomass or dissimilated into CO_2. Genome scale metabolic models combined with experimentally determined C_{eff} (carbon utilized for biomass and excreted products per methane consumed) provide a much better indication of how much carbon can be utilized. Methanotrophs grown in bioreactors have C_{eff} values of 31−61%, which supports the possibility that the calculated efficiency of 67% for methanotroph fuel production is reasonable.[32,164] By contrast, AOM assimilates 0.25−1.3% methane when coupled to sulfate reduction.[140,165] This low level of carbon assimilation is likely due to the poor energy yield of coupling methane oxidation to sulfate reduction rather than oxygen reduction and indicates that alternative electron acceptors will need to be investigated further. Although reverse methanogenesis pathways with CO_2, acetate, and biomass as products are thermodynamically feasible using all observed AOM electron acceptors, updated metabolic models underscore the importance of the reduction potential of the acceptor.[166] In addition, model scenarios in which bicarbonate is co-utilized with methane were shown to improve carbon efficiencies. Finally, this model shows methanol, ethanol, butanol, and isobutanol are thermodynamically feasible products.

An additional aspect of energetics that is not accounted for by E_{eff} is that the identity of the reductant used to support aerobic methane oxidation affects the efficiency of the respiratory chain. There is clear evidence that sMMO uses NADH.[82,83] The native reductant for pMMO is often suggested to be quinol based on assays performed *in vitro*.[167,168] Recent metabolic reconstructions of *Methylomicrobium buryatense* 5GB1 support the possibility that electrons from methanol dehydrogenase (MDH) via cytochrome c_L could be directly coupled to methane oxidation,[33,169,170] or that methanol oxidation partially supports methane oxidation via a mechanism referred to as the uphill model.[33,170] Importantly, the model shows that these possibilities could affect carbon utilization by more than 20%.[33] Also relevant to this model, electron microscopy and biolayer interferometry studies show MDH and pMMO interact directly.[169,171] This interaction could facilitate direct coupling of methanol oxidation to methane oxidation. Regardless of the exact reductant used by pMMO, a process based on sMMO is expected to be less efficient because reducing equivalents consumed at the level of NADH result in fewer protons being pumped. The difference is at least two protons if pMMO utilizes protons from the periplasm, but could be substantially higher if pMMO utilizes cytoplasmic protons or reductant that is higher potential than quinol. Interestingly, cells grown under low copper conditions assimilate 10% less carbon than cells grown under high copper conditions.[164] This difference is potentially attributable to the difference in reductants used by sMMO and pMMO, and also provides additional justification for using enzyme productivity as metric for comparing methane oxidation rates.

Complexity, Recombinant Tractability, and Overall Suitability for Bio-GTL. For Bio-GTL to be realized, either methane-oxidizing enzymes will need to be expressed in traditional hosts, or organisms that oxidize methane will need to be engineered to express downstream enzymes for fuel production. Methane-oxidizing enzymes are complex, and expression at high levels with correctly assembled metal cofactors presents a challenge that, for reasons detailed below, may be best met by expression in native organisms. However, methods for convenient genetic manipulation of methane-oxidizing organisms are in their infancy, and use of traditional laboratory strains such as *E. coli* or yeast that are well suited for high-throughput workflows and are better studied may be desirable.[172−174] Regardless of host, engineered strains will need to maintain high levels of active MMO/MCR expression or methane-oxidizing enzymes will need to be engineered to be faster to have carbon uptake rates similar to

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glucose uptake rates. If pMMO or MCR is used, better than wild-type expression levels or rates will be needed. Thus, the ability to use a native versus a recombinant host for Bio-GTL or protein engineering depends on which methane-oxidizing enzyme is being used and the intended application.

In terms of complexity, sMMO is the most tangible target and has the added advantage of being the fastest of the three methane-oxidizing enzymes. Several homologues, including toluene monooxygenase and phenol monooxygenase, have been successfully expressed in *E. coli*.[175,176] In addition, sMMO mutants have been successfully reintroduced into methanotrophs.[173,177−179] However, while the MMOR and MMOB proteins have been produced in *E. coli*,[180] heterologous expression of MMOH from *M. trichosporium* OB3b has been limited to strains of *Pseudomonas*,[181] which are capable of degrading trichloroethylene at 12.5% and 1.7% of the rates of *M. trichosporium* OB3b cells expressing sMMO with and without reductant added, respectively.[158,181] Methane oxidation by *Pseudomonas* strains expressing sMMO has not been demonstrated. Given that homologues of MMOH have been expressed in *E. coli*, the difficulties are likely not related to iron cofactor reconstitution, but perhaps to improper folding and assembly of the subunits. A high-throughput approach for improving MMOH expression in *E. coli* either through protein engineering or by testing sequences from newly discovered organisms would be a rational strategy for developing sMMO-based recombinant technologies. Alternatively, strains of *Pseudomonas* could be optimized for methane oxidation. Since it is unlikely stringent copper-free conditions can be maintained in an industrial process, use of a native host expressing sMMO would require either disruption of the copper switch or use of a methanotroph that only has the genes for sMMO.[58,182,183]

By contrast, pMMO is more challenging because it must be correctly incorporated into the membrane. The periplasmic domains of PmoB must be exported across the inner membrane and oriented correctly with respect to PmoA and PmoC.[50,96−98] The N-terminus of PmoB contains a signal peptide for export, which facilitates this process, ensuring that the N-terminus of PmoB is in the periplasm and the N-termini of PmoA and PmoC are in the cytoplasm.[184] Organisms expressing pMMO also form extensive ICMs, which can contain up to 80% pMMO[185] by protein content.[51,52,57,186] It is not known how these ICMs form and whether they are connected to the inner membrane. It is also unclear if the ICMs are simply present to increase the intracellular concentration of pMMO or whether they play a more complex role, such as concentrating methane. pMMO has been expressed in *Rhodococcus erythropolis* LSSE8-1, which does not form ICMs.[187] The whole cell activity of *Rhodococcus erythropolis* LSSE8−1 expressing pMMO is ∼340 times less than that of *Methylosinus trichosporium* OB3b, but this strain could sustain growth on ethane using endogenous pathways for ethanol assimilation. Thus, it may be that ICMs are not absolutely critical. However, the specific activity and expression level of this recombinant pMMO were not reported, so the cause of the low activity is not clear.

Another challenging aspect of recombinant pMMO expression is correct assembly of the copper active site. Copper is toxic to most microorganisms, which typically have multiple systems for its efflux and detoxification.[188,189] The concentration of copper in the growth medium has a dramatic impact on the activity of pMMO,[57,61,159] but very little is known about how the pMMO active site is assembled and how methanotrophs maintain appropriate intracellular copper levels. The genomes of methanotrophs encode a number of periplasmic copper binding proteins and in some cases, the copper homeostasis proteins CopC, CopD, and homologues of PCu_AC (DUF461) are found within the pMMO operon.[58,190−192] These proteins may play a role in pMMO active site assembly. Notably, extensive qRT-PCR studies of *M. trichosporium* OB3b indicate that the *copC*, *copD*, and *DUF461* genes are co-regulated with genes encoding pMMO and that a *copD* mutation is linked to constitutive expression of sMMO.[58] These findings establish a link between pMMO and copper homeostatic mechanisms, but the underlying interactions are not yet understood.

Beyond copper cofactor assembly and the role of ICMs, there remain considerable unknowns regarding pMMO function. Isolation of methanotroph membranes typically leads to a 10−100-fold decrease in pMMO activity, and solubilization followed by purification often results in almost complete inactivation of pMMO.[7] While removal from the native membranes could abrogate activity, it might also be that a yet-to-be-identified component of pMMO is lost or damaged during isolation. Such a component may be related to the electron-transfer pathway, which has not been fully resolved, or to helix X found in the crystal structures of pMMO,[96−98] and could be regulatory like MMOB or otherwise stabilize pMMO. In addition, the function of the conserved metal binding residues in PmoC has not been elucidated.[97,193] These sites may bind additional cofactors not present in the crystal structure. Additional studies are clearly needed to investigate these possibilities, especially if expression of pMMOs outside of their native hosts is desired.

Compared to methanotrophy, the cofactors used in AOM are biosynthetically much more complex, and ANME are much less tractable than methanotrophs. All three of the cofactors used by MCR, F_{430}, CoM, and CoB, are almost exclusively found in methanogens and ANME, and require significant biosynthetic machinery, which has not been fully characterized.[194−199] Thus, biosynthesis of these cofactors in hosts like *E. coli* or yeast is currently not possible. Furthermore, ANME cannot be isolated in pure culture let alone be genetically manipulated.[28] One strategy for using ANME MCR is to express it in a methanogen, which would have all of the biosynthetic machinery to make the required cofactors. This approach has recently been demonstrated using *Methanosarcina acetivorans*.[43] The resulting organism grew as a pure culture and was capable of oxidizing methane to acetate using carbonate as a cosubstrate and $FeCl_3$ as an electron acceptor. Although the rate of methane oxidation was slow, 28.6 μmol day^{-1}, this is the first genetically tractable system for producing ANME MCR.

In contrast to native enzymes that oxidize methane, spmoBs and CYPs are attractive because of their simplicity and amenability to recombinant expression and engineering. These advantages are far outweighed by several major issues, however. First, the spmoB and CYP activities will need to be improved by 3−4 orders of magnitude. Second, ways to complete their redox cycles based on biological reductants must be identified. In particular, spmoB will need to be engineered to utilize a different reductant because as a soluble protein, it will not have access to the quinone pool. Additionally, since spmoB expresses as inclusion bodies in *E. coli*, its solubility will have to be improved.[49,107] In the case of CYP, the enzyme would need to be engineered to be a true monooxygenase instead of generating compound I with iodosylbenzene.[108] Overall, there

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are major obstacles to using either of these proteins in a Bio-GTL process.

OUTLOOK

Bio-GTL is an immense economic opportunity that will need to employ one of the five known enzymes that oxidize methane. Each of these enzymes has its pros and cons. Long-term ANME MCR-based technology holds the most potential because it is expected to have higher carbon and energy efficiencies. However, there are several barriers that render technology development based on ANME MCR particularly difficult. First, ANME MCR is the most complex methane-oxidizing enzyme in terms of cofactors, the least recombinantly tractable, and has not even been isolated in a purified active form. Similarly, ANME are the least tractable methane-oxidizing organisms, and native AOM is far less understood than aerobic methanotrophy. Expressing ANME MCR in methanogens[43] is an important step toward developing industrial AOM pathways, but mastering the thermodynamics of these pathways will be a considerable challenge. Choosing the correct electron acceptor to ensure high carbon efficiencies will be critical as will be creating an efficient regeneration cycle for the electron acceptor. Along these lines, a thorough analysis of the economics of potential electron acceptors should be performed. Finally, MCR rates will need to be improved by at least 2 orders of magnitude to be comparable to sugar metabolism.

In the short term, aerobic pathways are more promising despite having lower carbon and energy efficiencies. Significantly more is known about methanotrophy, and initial pathways for methanotrophic production of fuels and chemicals have already been demonstrated.[35,36] Likewise, there is a current renaissance occurring in the development of genetic tools for these organisms, and MMOs are 1−2 orders of magnitude faster than ANME MCR (Table 2). Furthermore, sMMO is extremely well characterized, and there is a strong base of knowledge regarding pMMO. Thus, the pros and cons of employing sMMO versus pMMO are known. sMMO is less complex than pMMO and is more promising enzyme if a heterologous host is being used. However, pMMO is likely a better candidate for approaches that focus on engineering a methanotrophic strain since the carbon efficiencies are expected to be better as a result of using a higher potential electron donor. sMMO is faster than pMMO, but this advantage is partially offset by the improved energetics of pMMO and the lower cost of producing pMMO based on their calculated productivities. While sMMO and pMMO are slow enzymes, methanotrophs overcome this limitation by highly expressing them, resulting in carbon uptake rates that are similar in magnitude to glucose uptake rates in *E. coli*. Thus, MMOs may be fast enough for economical industrial scale production if large quantities of the enzyme can be produced cheaply and activity maintained for significant periods of time. To understand the upstream problem created by MMOs, current efforts should focus on determining and improving the lifetime of these catalysts and determining if Bio-GTL is sensitive to the cost of producing methane-oxidizing enzymes. Improving the rates of MMOs by at least 1 order of magnitude would improve the viability of Bio-GTL processes and should be pursued alongside improving the overall growth parameters of methanotrophs, designing efficient fuel-producing pathways, and improving bioreactor design. For now, it is all about methane-oxidizing enzymes and organisms, but the day when Bio-GTL is "all about the Benjamins" may not be far off.

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Notes

The authors declare no competing financial interest.

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