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INVITED REVIEW PAPER

Biological conversion of methane to methanol

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Abstract–The conversion of methane to methanol is important to economic utilization of natural/shale gas. Methanol is a valuable liquid fuel and raw material for various synthetic hydrocarbon products. Its industrial production is currently based on a two-step process that is energy-intensive and environmentally unfriendly, requiring high pressure and temperature. The biological oxidation of methane to methanol, based on methane monooxygenase activity of methanotrophic bacteria, is desirable because the oxidation is highly selective under mild conditions, but conversion rate and yield and stability of catalytic activity should be improved up to an industrially viable level. Since methanotrophic bacteria produce methanol as only a precursor of formaldehyde that is then used to synthesize various essential metabolites, the direct use of bacteria seems unsuitable for selective production of a large amount of methanol. There are two types of methane monooxygenase: soluble (sMMO) and particulate (pMMO) enzyme. sMMO consisting of three components (reductase, hydroxylase, and regulatory protein) features an $(\alpha\beta\gamma)_2$ dimer architecture with a di-iron active site in hydroxlase. pMMO, a trimer (pmoA, pmoB, and pmoC) in an $\alpha_3\beta_3\gamma_3$ polypeptide arrangement is a copper enzyme with a di-copper active site located in the soluble domain of pmoB subunit. Since the membrane transports electrons well and delivers effectively methane with increased solubility in the lipid bilayer, pMMO seems more rationally designed enzyme in nature than sMMO. The engineering/evolution/modification of MMO enzymes using various biological and chemical techniques could lead to an optimal way to reach the ultimate goal of technically and economically feasible and environmentally friendly oxidation of methane. For this, multidisciplinary efforts from chemical engineering, protein engineering, and bioprocess research sectors should be systematically combined.

Key words: Natural/Shale Gas, Methane, Methanol, Biological Oxidation, Methane Monooxygenase

INTRODUCTION

Worldwide reserves of natural gas were recently estimated at about 208.4 trillion m^3 [1]. This figure continues to increase as new deposits are discovered, and consumption continues to rise. Shale gas - natural gas formed and trapped within shale formations - is rapidly becoming an alternative source of natural gas and its production is one of the fastest-growing segments of the U.S. oil and gas industry today. Natural gas production in the U.S. will be increasingly driven by shale gas production. The U.S. Energy Information Administration's Annual Energy Outlook 2012 [2] predicts an increase in shale gas production from 5.0 trillion ft^3/yr in 2010 to 13.6 trillion ft^3/yr in 2035, corresponding to an increase from 23% to 49% of the total U.S. dry gas production, respectively.

Natural/shale gas contains methane as a primary component. Methane can either be used as a source of fuel in its own right, as a chemical precursor by the chemical and petrochemical industries, or as a reducing agent in the iron ore conversion process by the iron and steel industry. However, methane occurs as a gas at ambient temperatures (boiling point: −164 °C), making it expensive to transport from where it naturally occurs in remote locations to where it will be consumed [3]. Another disadvantage is that methane is a potent greenhouse gas, with a global warming potential more than 20 times that of carbon dioxide [4].

One way of addressing these challenges is to transform methane to methanol. Efforts to do so cost-effectively in an environmentally friendly way are attracting considerable attention from industries. The petrochemical industry uses methanol as raw material for the production of various chemicals such as MTBE, formaldehyde, acetic acid and biodiesel via trans-esterification [5]. Apart from the fact that methanol can be used as a fuel on its own, it can also be used in fuel mixtures such as with gasoline. Methanol also has been successfully used in fuel cells to generate electricity, which is a process that involves the electrochemical oxidation of methanol with air, releasing carbon dioxide and water as the only by-products [6]. Methanol serves as a raw material for many synthetic hydrocarbons and their products; for example, it can be conveniently converted into ethylene or propylene through the methanol-to-olefins process [6]. The production of these olefins is an alternative route to hydrocarbon fuels and their products, which are presently obtained from oil and gas [6].

Although the chemical process to convert methane to methanol has been intensively studied for several decades, several disadvantages remain still unsolved. It relies on a metal catalyst and requires extreme energy-intensive conditions to achieve the conversion that usually accompanies production of synthetic gases, hence indicating an environmentally unfriendly process. In contrast, biological conversion using methane monooxygenase (MMO) enzyme involves a simple and highly selective reaction under mild conditions. However, the biological process poses some challenges, notably, slow reaction rate, chemical instability of enzyme, and higher cost associated with the use of enzyme. In this article, we will review the critical issues relating to the chemical and biological conversion of

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Table 1. Catalysts for direct partial oxidation, methane to methanol

Catalyst	Pressure	Temp. (°C)	Selectivity (%)	Oxidant	Methane conversion (%)	Ref.
Cu/SiO_2 (10%)	30 atm	300-357	-96	O_2	6	[9]
SnO_2	30 atm	247-307	27-83	O_2	1-4	
Hg(II)	-	180	85	H_2SO_4	50	[3]
$MoO_3 : UO_2$	5.4 MPa	388-543	23-49	O_2	1-5	[10]
Nafion-H/teflon/C	-	120	100	H_2O_2	-	[11]
Fe Sodalite	-	435	25	O_2	5.8	
MoO_3	15 bar	450	11	O_2	0.3	[12]
Cu/MoO_3	15 bar	450	19	O_2	0.6	
Ga_2O_3/MoO_3	15 bar	455	22	O_2	3.0	
$PMo_{11}V$	1 atm	700-750	12.25-19.89	O_2	3-13	[13]
$PMo_{11}Fe$	1 atm	700-750	36.6-36.72	O_2	4-23	
$SiMo_{11}Fe$	1 atm	700-750	28.31-29.68	O_2	4-32	
ZSM-5	30.5 bar	50	19	H_2O_2	0.3	[14]
Al/ZSM-5	30.5 bar	50	22	H_2O_2	0.2	
Cu-Fe/ZSM-5	30.5 bar	50	85	H_2O_2	0.7	
Fe-silicalite and Cu/silicalite-1	3 bar	70	93	H_2O_2	10.1	
$AuPd/TiO_2$	30.5 bar	2-70	29.5-89.2	H_2O_2, H_2/O_2	-	[15]
Au_2O_3	27 bar	180	>90	SO_3	-2	[16]
Au+additives ($H_2SeO_4/SeO_4/O_2$)	27 bar	180	81-94	SO_3	3-28	

methane to methanol, more focusing on the use of biological methods to solve the traditional problems associated with chemical transformation.

CHEMICAL OXIDATION OF METHANE TO PRODUCE METHANOL

Methane is a very unreactive molecule because of its high C-H bond strength [D(C-H)=438.8 kJ/mol], high ionization potential (12.5 eV), low proton affinity (4.4 eV), and low acidity (pKa=48) [7,8]. The methane molecule can be activated by using very reactive species involving various catalysts, a number of which have been studied to date [3,9,10-16] (Table 1). The design of the catalytic cycle is also an essential step in the oxidation of methane to methanol [7]. However, all these chemical reactions are usually energy-intensive, and as such, environmentally unfriendly. Nevertheless, methanol is mostly produced by this method on account of economic feasibility. In general, two methods are commonly used to convert methane to methanol: a two-step process and a direct conversion method.

1. Two-step Process

The most common way of producing methanol from natural gas is a costly two-step process (Fig. 1(a)) [17]. It involves the production of synthetic gas (syngas) by treating methane with steam, followed by the conversion of the syngas to methanol [5]. According to a conventional scheme [17,18], the first step is energy-intensive

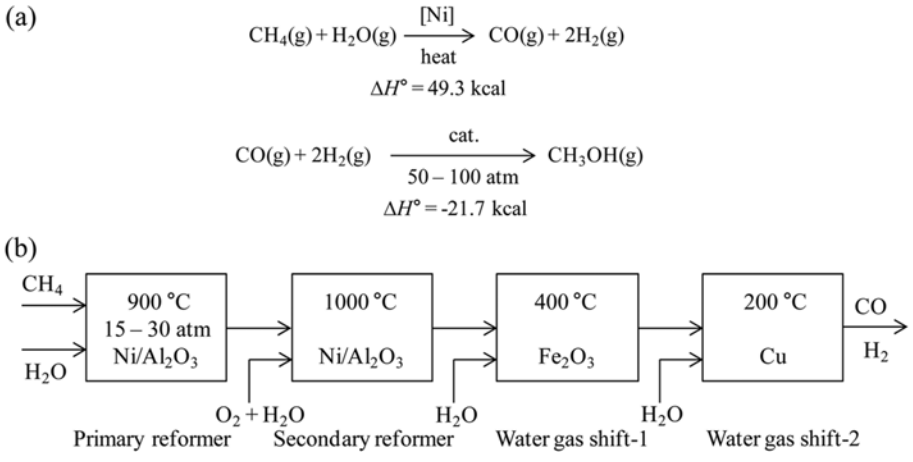

Fig. 1. (a) Two-step methanol formation reactions [17]. (b) Block diagram for conventional steam reforming [18], consisting of a primary reformer where CH_4 and excess H_2O are reacted over Ni/Al_2O_3, at 900 °C, a secondary reformer where the unconverted CH_4 (8-10%) is reacted with O_2 and more H_2O to give equilibrium CO and H_2, and two water gas shift stages where the product H_2/CO ratio is then modified.

conversion of methane using steam that generates a substantial amount of greenhouse gases, requiring a catalyst and high temperature in the range of 800-1,000 °C (Fig. 1(b)), and the second step to produce methanol from syngas requires a high pressure of 50 bar and a temperature of 250 °C. As an effort to simplify the conversion process with the reduction of energy dependence, there is ongoing research to achieve direct partial oxidation under relatively mild conditions [19].

2. Direct Conversion Method

The direct conversion of methane to methanol in a single step is clearly advantageous. A variety of catalysts, reactors, and oxidants have been studied to date [20]. Unfortunately, no process is capable of producing methanol in a reasonable yield, but there has been encouraging progress, including homogeneous oxidation that occurs in the pure gas phase by using an effective catalyst [21]. Reportedly, catalysts with mainly metal surfaces such as zinc, nickel, copper, silver, steel, and certain alloys have been used with success [22]. However, the partial oxidation of methane in a thermal reactor occurs under conditions that also induce methanol oxidation. This leads to the formation of carbon oxides and lowers the selective formation of methanol. Reportedly, methanol yield is very low (e.g., less than 2% [24]), which is far less than the yield (>10%) that would be required to make industrial production economically feasible [23, 24].

Of the more recent techniques considered for the production of methanol, the non-thermal dielectric barrier discharge (DBD) plasma chemical process is considered to be one of the most promising [5, 25]. A DBD reactor (Fig. 2) relies on the application of a high voltage to a pair of grounded electrodes separated by a dielectric substance such as quartz. The resulting discharge contains excited, ionized, metastable compounds and radicals that can be generated by the collision of energetic electrons and reactant particles. These energetic electrons have a higher temperature than the background gas, resulting in a non-equilibrium plasma that lowers the reaction temperature to room temperature. In terms of achieving mild reaction conditions, the DBD plasma process therefore holds more promise than catalytic conversion in a thermal reactor [24-26].

NATIVE BIOCATALYSTS TO CONVERT METHANE TO METHANOL

The ongoing discovery of new natural gas reserves and the increasing demand for energy sources call for more efficient ways to convert methane into methanol with less damaging to the environment. To avoid the extreme operating conditions required by conventional metal catalysts, the development of new catalysts capable of efficiently oxidizing methane under mild conditions has become a high priority of industry. The development of efficient biocatalysts to convert methane to methanol could have significant implications for the use of methane as an alternative energy source and chemical feedstock [27]. A possible way for biological methane oxidation is to use methanotrophic bacteria (i.e., methanotrophs) that are capable of activating the stable C-H bond under ambient conditions. These bacteria contain a special enzyme, methane monooxygenase (MMO) that is used to utilize methane as a sole carbon and energy source under aerobic conditions [4]. The detailed characteristics of Methanotrophs and MMO were summarized below, which may be very useful to the development of novel and efficient biocatalysts.

1. Methanotrophic Bacteria

Although methanotrophs have been known for nearly 100 years, they were successfully isolated by Whittenbury and coworkers in 1970 [28]. Methanotrophs can grow aerobically or anaerobically and use methane as a sole source of carbon and energy. They are found in a variety of habitats such as mud, swamps, rivers, rice paddies, streams, oceans, ponds, meadow soils, sediments, deciduous woods, and sewage sludge [29]. Different strains of the bacteria have also been found capable of thriving under extreme conditions, for example, in the acidic peat wetlands in northern Europe and Siberia [30], in alkaline lakes in central Asia [31] and Kenya [32], in hot springs in Hungary [33], and in saline lakes in Antarctica [34]. Methanotrophs metabolize methane by oxidizing it to methanol using MMO enzymes under aerobic conditions, and then methanol is converted to formaldehyde by methanol dehydrogenase, which is used to synthesize various multi-carbon compounds or subsequently oxidized to formate by formaldehyde dehydrogenase (Fig. 3) [27].

Methanotrophs are classified into three types on the basis of their carbon assimilation pathways, cell morphology, membrane arrangement, 16S RNA sequences, and other metabolic characteristics [4, 29,35-42] (Table 2). The type I methanotrophs, including genera *Methylomonas*, *Methylobacter*, *Methylomicrobium*, *Methylosphaera*, and *Methylocaldum*, use ribulose monophosphate (RuMP) pathway to metabolize formaldehyde, whereas the type II methanotrophs, such as genera *Methylosinus* and *Methylocystis*, rely on serine pathway utilizing ribulose bisphosphate carboxylase to effect carbon assimilation. The type X methanotrophs, such as *Methylococcus*, use a combination of both of these metabolic pathways although they mainly rely on the RuMP pathway (Table 2, Fig. 3) [27] and thrive at higher temperatures than type I or type II methanotrophs. The detailed features that distinguish the three types of methanotrophs are described more in previous review articles [27,29].

2. Methane Oxidation Enzyme - MMO

Methanotrophs metabolize methane by oxidizing it to methanol,

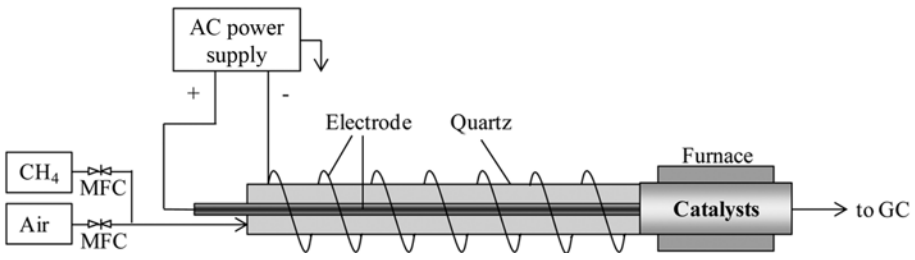

Fig. 2. Scheme diagram of a DBD reactor and experimental setup [25].

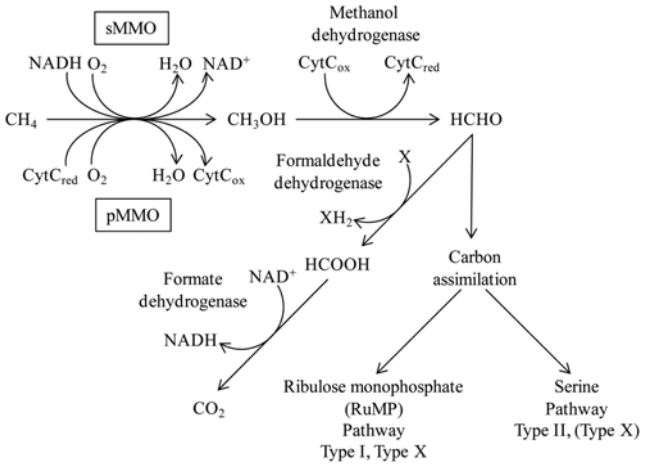

Fig. 3. Metabolic pathways of Methanotrophs (CytC represents cytochrome C) [27,29].

requiring MMO enzymes as catalyst under aerobic conditions. As shown in Table 3 [43], MMO is capable of oxidizing a broad range of alkanes besides methane, indicating unusually low substrate specificity. There are two types of MMOs: soluble and particulate forms. The soluble form (sMMO) occurs in bacterial cytoplasm under conditions with a low concentration of copper [44]. The more common membrane-bound form, referred to as particulate MMO (pMMO) [27,45,46], requires a copper-saturated environment to actively function [4,47]. Of the two types of MMOs, most researchers have studied first sMMO that can be much more easily isolated than pMMO, even though pMMO is more abundant in nature. sMMO consists of three components: a hydroxylase (MMOH), a reductase (MMOR), and a regulatory protein (MMOB), and its biochemical properties, structure, and activity mechanism have been described previously in detail [27]. Compared to sMMO, pMMO is not so well understood and has not yet been reviewed comprehensively. A better understanding of the basic mechanisms for enzymatic conversion of methane to methanol would enable one to design and engineer more efficient biocatalysts.

2-1. sMMO

To date, two methanotrophs, *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b, have mainly been used for sMMO studies. Patel et al. [48] separated the three different components of sMMO using ion exchange chromatography and found that maximum enzyme activity was only achieved by combining all three fractions. Green and Dalton [49] further investigated the function of sMMO components using *M. capsulatus* (Bath) and showed that sMMO is a multi-component enzyme consisting of a hydroxylase (MMOH), a reductase (MMOR), and a regulatory component (MMOB), all three of which being required for monooxygenase activity. They also found that even when the regulatory component is absent, electron transfer can occur between hydroxylase

Table 3. Oxidation of C_1-C_8 n-alkanes by sMMO from *M. capsulatus* (Bath) [43]

Substrate	*M. capsulatus* (Bath)	
	Products	Specific activity (milli-units/mg of protein) 1 unit=1 μmol of product/min
Methane	Methanol	84
Ethane	Ethanol	68
Propane	1-Propanol	69
	2-Propanol	
Butane	1-Butanol	77
	2-Butanol	
Pentane	1-Pentanol	73
	2-Pentanol	
	3-Pentanol	
Hexane	1-Hexanol	40
	2-Hexanol	
	3-Hexanol	
Heptanes	1-Heptanol	27
	2-Heptanol	
	3-Heptanol	
	4-Heptanol	
Octane	1-Octanol	9
	2-Octanol	
	3-Octanol	
	4-Octanol	

Table 2. Classification of methanotrophic bacteria [4]

Type	Metabolic pathway for carbon source	Genera	Genome sequenced	Ref.
Type I	Ribulose monophoshpate pathway (RuMP)	*Methylomicrobium*, *Methylobacter*, *Methylomonas*, *Methylosphaera*, *Methylocaldum*	*Methylomicrobium alcaliphilum* 20Z	[35]
Type II	Serine pathway	*Methylocystis*, *Methylosinus*, *Methylocella*	*Methylosinus trichosporium* OB3b *Methylocystis* species *Methylocella silvestris* BL2	[36][37][38] [39][40][38] [41]
Type X	RuMP Low levels of serine pathway enzymes	*Methylococcus*	*Methylococcus capsulatus* (Bath)	[42]

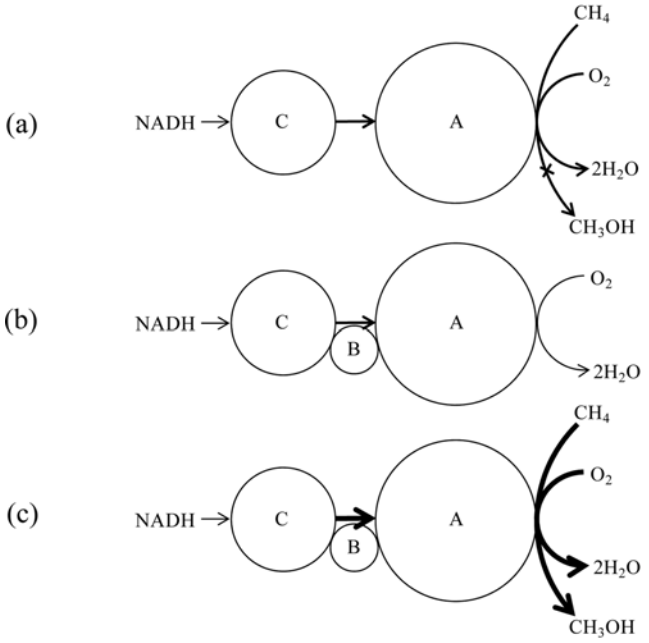

Fig. 4. Effect of MMOB on electron transfer within sMMO (A, B, and C represent MMOH, MMOB, and MMOR, respectively). (a) Without MMOB, MMOH and MMOR only catalyze oxygen reduction to water in the presence or absence of CH_4. (b) Addition of MMOB switches the enzyme complex from an oxidase to an oxygenase. Without CH_4 steady-state electron transfer between MMOH and MMOR is shut down. (c) Addition of CH_4 to the complete sMMO complex restores electron transfer, and the methane oxidation is catalyzed (The width of the arrows reflect the relative rate of the reaction indicated) [49].

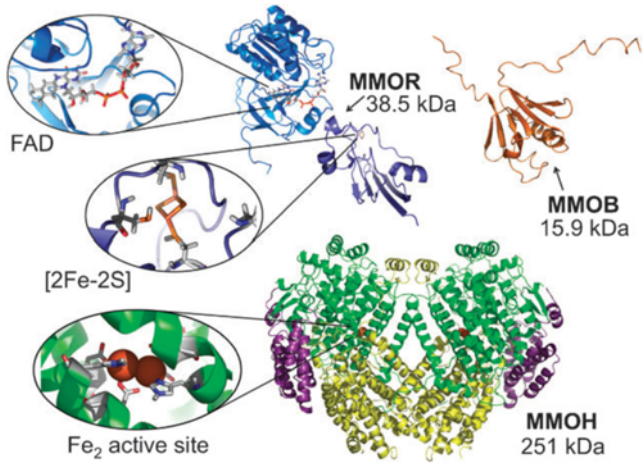

Fig. 5. sMMO enzyme system from *Methylococcus capsulatus* (Bath) consists of three components: 1) a hydroxylase [MMOH (PDB reference 1MTY)], 2) an oxidoreductase [MMOR consisting of FAD domain (PDB reference 1TVC) and [2Fe2S]-Fd domain (PDB reference 1JQ4)], and 3) a regulatory (binding) protein [MMOB (PDB reference 1CKV)]. The ribbon diagram of MMOH is based on X-ray coordinates, and those of MMOB and the two truncated MMOR fragments are based on NMR structures [51].

and reductase, but that the regulatory protein is essential to preventing the steady transfer of electrons in the absence of methane. They finally demonstrated that the active site of the hydroxylase is the catalytic center where the activation/reduction of O_2 and the oxidation of CH_4 take place and proposed a mechanism to explain the function of sMMO, as illustrated in Fig. 4 [49]. The detailed physical properties of purified sMMO are well summarized in a previous publication [50].

As presented in Fig. 5 [51], sMMO has a dimeric structure made up of two $\alpha\beta\gamma$-protomers, featuring an $(\alpha\beta\gamma)_2$ dimer architecture. Each protomer has an almost entirely α-helical secondary structure. Each of the two identical α-subunits contains a four-helix bundle which serves for the di-iron active site that performs the hydroxylation. The structure and reactivity of the di-iron site of MMOH is altered by MMOB, a protein cofactor required for the activation of MMOH. MMOR contains a bound flavin adenine dinucleotide (FAD) and a [2Fe-2S]-ferredoxin (Fd) cofactor and transfers electrons from NADH/NADPH (electron donors) to MMOH (a carboxylate-bridged di-iron center that is located in a four-helix bundle of the α-subunit within MMOH). Therefore, three components, MMOH, MMOR, and MMOB, should be assembled to form the whole enzyme system for methane oxidation via the selective transportation of the four substrates (methane, oxygen, electrons, and protons) to the active

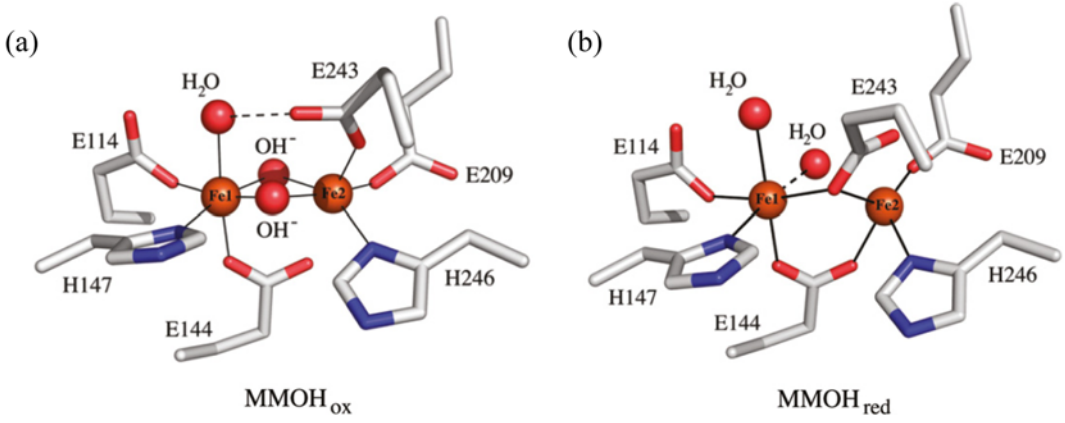

Fig. 6. Di-iron active site structures of (a) oxidized state of MMOH ($MMOH_{ox}$ (PDB reference 1MTY) and (b) reduced state of MMOH ($MMOH_{red}$ (PDB reference 1FYZ) [52].

di-iron site [51].

The detailed mechanism of methane oxidation by sMMO was already discovered at molecular level, as illustrated in Fig. 6 [51,52]. The active di-iron site forms the complex consisting of two histidine (H147 and H246) and four glutamate (E114, E144, E209, and E243) ligands around two Fe ions [52]. The pseudo-octahedral coordination spheres contain solvent molecules (H_2O) on the outer perimeter. Two hydroxide ligands bridge the iron atoms, which are 3.1 Å apart in the inactive state (Fig. 6(a)). When the di-iron is reduced to form $MMOH_{red}$, the bridging hydroxide ligands depart, and E243 is presumably located at a bridging position (Fig. 6(b)). This causes the Fe-Fe distance to increase and opens up a coordination site, enabling O_2 to react with the reduced di-iron center of MMOH. The breaking of the O-O bond causes the di-iron core to rearrange itself, followed by the oxidation of methane to methanol.

2-2. pMMO

The X-ray crystal structure of pMMO from *M. capsulatus* (Bath) has been determined: pMMO is a trimer (pmoA, pmoB, and pmoC) in an $\alpha_3\beta_3\gamma_3$ polypeptide arrangement, approximately 105 Å long and 90 Å in diameter [46]. Each of pmoA, pmoB, and pmoC consisting of three identical subunits, and each $\alpha\beta\gamma$ protomer houses three metal centers (Fig. 7, 8). The hydrophilic and soluble region of the enzyme, which extends ~45 Å from the membrane, is composed of six cupredoxin-like β-barrels, two from each of the pmoB subunits. The central opening in the soluble region of the pMMO trimer is approximately 11 Å wide and is lined with charged residues. pMMO also has a total of 42 transmembrane helices: each pmoB, pmoA, and pmoC subunit contains two, seven, and five helices, respectively. Another investigation of *M. capsulatus* (Bath) pMMO by electron microscopy (EM) reveals a protein complex consistent in size and shape with the crystal structure (Fig. 8(c)), suggesting that the trimeric arrangement observed in the crystal structure is physiologically relevant. An interesting feature from EM analysis, which is not observed in the crystal structure, is regions of low density or "holes" in the soluble regions of pmoB, which could possibly indicate sites of substrate entry or product exit (Fig. 8(c)) [46]. Although previous biochemical and structural studies were focused on pMMOs isolated from *M. capsulatus* (Bath) and *M. trichosporium* OB3b, Smith et al. [53] recently studied pMMO from *Methylocystis* sp. strain M and structurally characterized pMMO from this new organism, providing a revised model for the pmoA and pmoC subunits of *M. capsulates* (Bath) pMMO through revealing a different coordination environment for the intramembrane zinc/copper binding site.

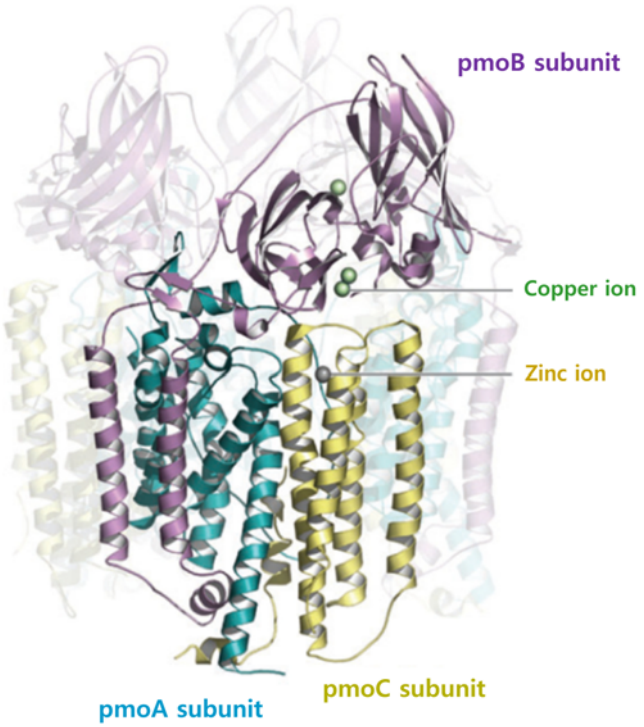

Fig. 7. pMMO trimer (PDB reference 1YEW) with one protomer highlighted [46].

The investigation of *M. capsulatus* (Bath) pMMO structure revealed the presence of three metal centers, including two copper centers in the soluble domain of pmoB subunit: 1) a mono-copper center (coordinated by His 48 and His 72) at the interface between the two cupredoxin domains, and 2) a di-copper center (coordinated by His 33, His 137, and His 139) close to the membrane interface (Fig. 9) [4,54]. The residue equivalent to His 48 is an Asn residue

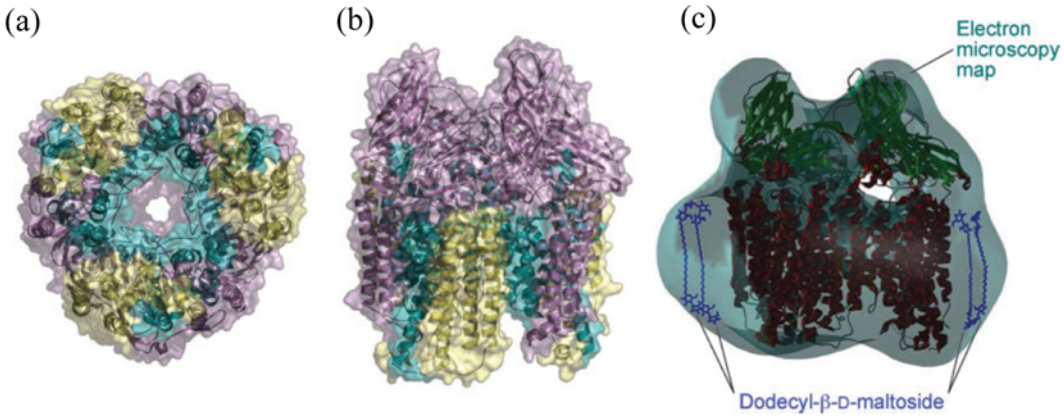

Fig. 8. Surface representations of pMMO viewed (a) perpendicular to and (b) parallel to the membrane normal, and (c) pMMO crystallographic coordinates and four molecules of dodecyl-β-d-maltoside that are used to extract pMMO from membrane (PDB reference 1QLA) docked into the electron microscopy map [46].

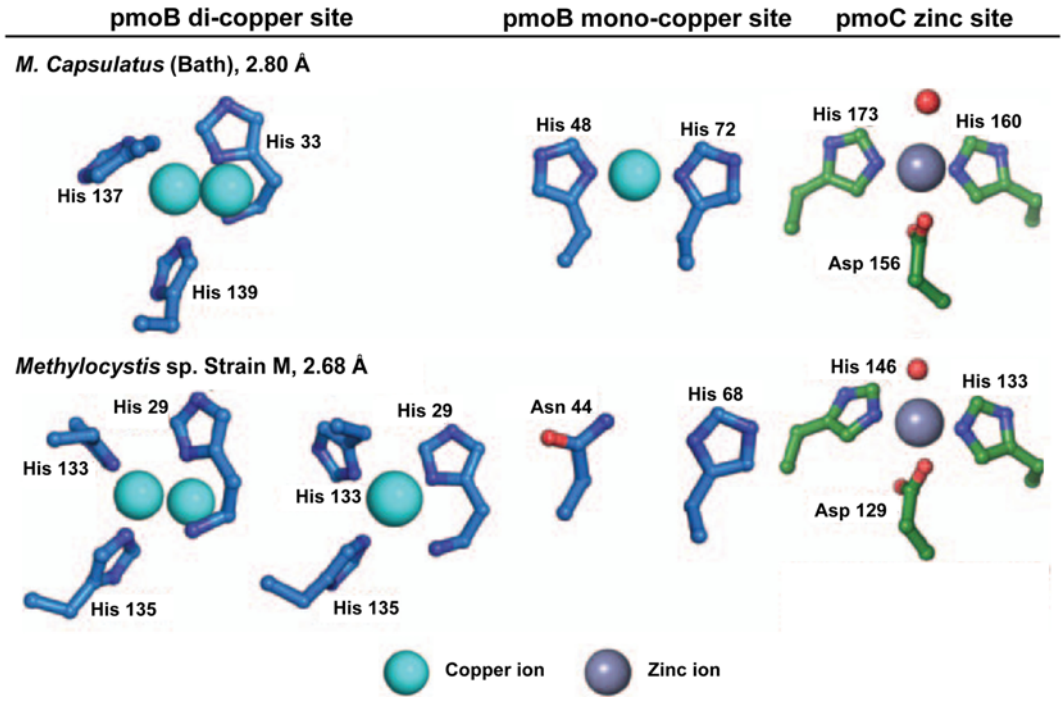

Fig. 9. Metal centers modeled in pMMO crystal structures [4].

of pMMOs both in the *M. trichosporium* OB3b and in *Methylocystis* sp. strain M, indicating that His 48 is not a conserved residue. Neither one of the strains contains copper at the center corresponding to the mono-copper center of *M. capsulatus* (Bath) pMMO (Fig. 9). Residue His 33 is the amino terminal residue of pmoB subunit (the first 32 residues are a signal sequence that is removed *in vivo*), with its amino group located within coordinating distance of the di-copper center (Fig. 9). With the exception of Verrucomicrobia, all methanotroph pmoB sequences contain these three histidine residues at the di-copper center. The third metal center was found to be occupied by zinc in the pMMO structures of *M. capsulatus* (Bath) and *Methylocystis* sp. strain M and found to be located within the membrane. Recently, Balasubramanian et al. [54] found through the analysis of recombinant soluble fragments of pMMO that pMMO activity is dependent on copper and the active site is a di-copper center. They resolved the previous pMMO controversy regarding which metal center is a true active site and concluded that pMMO is a copper enzyme with a di-copper active site located in the N-terminal soluble domain of pmoB subunit. However, it is not completely understood yet how the functions of other subunits within trimeric structure are correlated to maintain native pMMO activity, which is an important issue that should be disclosed in future.

BIOLOGICAL CONVERSION OF METHANE TO METHANOL

1. Utilizing Methanotrophic Bacteria as Biocatalyst

Initial investigators focused on the use of methanotrophs for biological oxidation of methane. The primary objective of these researches was to solve the greenhouse gas problem [55]. However, the conversion rate of methane to methanol was too low, and the growth conditions of bacteria were very complex [56]. An effective way proposed was to oxidize methane occurring in landfills by methanotrophic microorganisms in soils or waste materials utilizing oxygen from the atmosphere [57]. The intracellular reaction steps involving the microbial oxidation of methane to carbon dioxide by methanotrophs are shown in Fig. 3 [29], showing that the oxidation of methane to methanol is followed by oxidation of methanol to formaldehyde (CHOH) and the subsequent oxidation of formaldehyde to formate (CHOOH) [57]. In this metabolic pathway, methanol is not the final product and only a precursor of formaldehyde that is used to synthesize various metabolites that are essential to survival of bacteria, indicating that the use of native bacteria seems not suitable for selective production of a sufficient amount of methanol. Since genome sequencing of some methanotrophic bacteria has been completed (Table 2), it may be possible to construct genetically or metabolically engineered methanotrophs for overproduction of methanol; however, this looks like a highly challenging task with very narrow chance for success because methanol is an only precursor of formaldehyde that is used to synthesize various essential metabolites for bacterial growth.

2. Utilizing MMO as Biocatalyst

Compared to sMMO, the structure, function, and catalytic mechanism of which were uncovered in detail, pMMO has not been so intensively studied, because it is highly difficult to isolate and purify the membrane-bound particulate enzyme in the active form. A variety of analytical studies have been performed for the systematic and in-depth analysis of methane oxidation by sMMO and pMMO, the detailed review of which would be helpful in finding critical key issues that should be carefully considered upon utilizing MMOs as biocatalyst for the methane conversion to methanol.

For the catalysis of methane oxidation by sMMO, NADH is normally required as an electron donor (reductant), but the supply of a sufficient amount of NADH for large-scale process seems not practi-

cal on account of both technical and economic feasibility. Many researchers have used duroquinol (2,3,5,6-tetramethylhydroquinone) as the alternative to NADH [37,43,54] to support pMMO activity, but it is still too expensive for large-scale process (e.g., lowest Sigma price in USA: $ 13/g). Interestingly, Jiang et al. [58] replaced O_2 and NADH by H_2O_2 serving as the source of both oxygen and electrons for catalytic reaction of sMMO from *M. capsulatus* (Bath). Owing to the much cheaper price of H_2O_2 (e.g., lowest Sigma price in USA: $ 0.028/g), the use of H_2O_2 may significantly improve the cost-effectiveness of biological methane oxidation process, though methane conversion rate still remains a critical issue to be solved.

Jiang and Dalton [59] used proteotically modified hydroxylase (MMOH) of sMMO to oxidize ethane, propane, propene, and methane in an H_2O_2-driven reaction above and found that the proteolyzed hydroxylase retained full activity for ethane, propane and propene and showed even a 2-3-fold increase of activity when methane was used as substrate. They concluded that the part of the hydroxylase responsible for the interaction with H_2O_2 is unaffected by proteolysis and that the truncated form of the hydroxylase has a more accessible active site to methane than does the native form. The same researchers [59] also modified MMOH of sMMO using methoxypolyethylene glycol (methoxy-PEG) that has been successfully used for the modification of many enzymes (lipase [60-62], α-chymotrypsin [63-68], trypsin [67,69], thermolysin [70], papain [71-73], catalase [74], peroxidise [73,75-78], cholesterol oxidase [79]) because activated, or monofunctionally derivatized PEG reacts easily with amine residues of proteins. The activated PEG-modified MMOH was soluble even in organic solvents (dichloromethane, toluene, benzene, hexane, ethanol) as well as water, was activated by H_2O_2, and functioned well in the catalysis of propene oxidation. However, unfortunately, they failed in observing the catalytic activity of activated PEG-modified MMOH in methane oxidation. The proteolysis- and PEG-based modification of MMO above suggests that there may be an optimal way of engineering MMO to enhance its catalytic activity. Undoubtedly it will have a great impact on the industrial sector if a high rate of enzymatic methane oxidation is achievable using more practical electron donors like H_2O_2 or even in organic solvents in which methane solubility can be noticeably enhanced.

Glieder et al. [80] engineered cytochrome P450 BM-3 from *Bacillus megaterium* (P450 BM-3), i.e., a medium-chain (C_{12}-C_{18}) fatty acid monooxygenase, into a highly efficient catalyst for the conversion of alkanes to alcohols. The evolved P450 BM-3 exhibits higher turnover rates than any reported biocatalyst for the selective oxidation of hydrocarbons of small to medium chain length (C_2-C_8) and is more active even on fatty acids unlike naturally occurring alkane hydroxylases like MMO and AlkB (membrane-associated non-heme iron alkane monooxygenase). This strategy of enzyme evolution to find a novel biocatalyst with enhanced activity for alkane hydroxylation at room temperature may open new opportunities for clean and selective oxidation of hydrocarbons including methane.

Recently, Lee et al. [81] elucidated the role of MMOB to support sMMO activity, which has remained ambiguous owing to a lack of atomic-level information about the MMOH-MMOB complex. According to their findings, MMOB controls the conformation of residues in MMOH crucial for substrate access to the active site. That is, MMOB docks at the $\alpha_2\beta_2$ interface of $\alpha_2\beta_2\gamma_2$ MMOH and triggers simultaneous conformational changes in the α-subunit that modulate oxygen and methane access as well as proton delivery to the di-iron center. Without such careful control by MMOB in these substrate routes to the di-iron active site, the enzyme does not properly operate as a monooxygenase. Based on these results, it is now possible to delineate access pathways of methane to the active site of sMMO, which is highly important information that can be used for rational engineering of the sMMO system.

Maintaining stably the enhanced activity of MMO during methane oxidation is another critical issue to be solved to increase the enzymatic conversion rate up to a sufficiently high level for industrial application. Jiang and Dalton [59] chemically cross-linked the proteolyzed MMOH by reacting it with polyoxyethylene bis(imidazolyl carbonyl), resulting in modification of approximately 22% of the accessible amine groups in MMOH. This chemical cross-linking strengthened the compactness of MMOH structure due probably to intramolecular 'bracket' and accordingly increased thermostability of MMOH, so that denaturation of MMOH at high temperatures was significantly reduced. However, the cross-linking positions always vary in space and microenvironment, because the amino acid residues susceptible to cross-linking reagents are distributed randomly in three-dimensional enzyme structure, indicating that the chemical cross-linking method may cause serious reproducibility problems in modulating enzyme activity and stability.

Despite the recent findings by Balasubramanian et al. [54] that pMMO is a copper enzyme with a di-copper active site located in the N-terminal soluble domain of pmoB subunit, little is still known for pMMO, compared to sMMO. It is noticeable that Balasubramanian et al. [54] cloned and expressed in *Escherichia coli* the soluble cupredoxin domains of the pmoB subunit from *M. capsulatus* (Bath). The expressed recombinant pmoB domains include N-terminal cupredoxin domain (spmoBd1, residues 33-172), Gly-rich linker, and C-terminal cupredoxin domain (spmoBd2, residues 265-414), which was refolded in the presence of $CuSO_4$ from intracellular inclusion bodies. The activity of recombinant pmoB was about 60% of activity of as-isolated, membrane-bound native pMMO both in epoxidation of propylene to propylene oxide and in oxidation of methane to methanol. The lower activity of recombinant pmoB than as-isolated pMMO indicates that there must be an unknown functional correlation among the subunits within the trimeric structure of pMMO, which is an important issue awaiting future discovery.

According to the report by Choi et al. [82], pMMO activity was stimulated by either copper ion or copper-methanobactin (Cu-mb) that is contained in the washed membrane fraction of *M. capsulatus* (Bath) cultures. It has been suggested that mb may initially bind copper as a homodimer, i.e., as $Cu(mb)_2$, followed by the binding of a second copper, resulting in a final molar ratio of 1 copper atom per mb, i.e. Cu-mb. The stimulation of pMMO activity by Cu-mb in washed membrane fractions increased proportionally with the copper concentrations used during the growth of *M. capsulatus* (Bath), indicating that copper concentration is of crucial importance to enhancing pMMO activity. Yu et al. [83] prepared pMMO-enriched membranes from *M. capsulatus* (Bath) with high activity and in high yields, using a fermentor adapted with a hollow-fiber bioreactor where the copper ion concentration was quantitatively adjusted in growth medium over the time course of cell culture. They determined an optimal copper concentration in the growth medium (30

to 35 μM) and discovered that 80% of the total cytoplasmic membrane proteins were pMMO, indicating again the critical effect of copper ion on active pMMO synthesis.

Gou et al. [84] synthesized recombinant pMMO in *Rhodococcus erythropolis* LSSE8-1 transformed with an expression vector containing the structural gene cluster pmoCAB of pMMO from *M. trichosporium* OB3b. *R. erythropolis* LSSE8-1, originally isolated from soil in a natural gas well for petroleum biodesulfurization, cannot utilize hydrocarbons such as methane, ethane, dodecane and hexadecane, showing a useful trait because it does not reduce the combustion value of petroleum. This strain, however, can use ethanol as the sole carbon source for growth, and hence the transformant with pMMO function can oxidize ethane into ethanol, which can then be utilized further for cell growth. They observed that the pmo genes were transcribed in the recombinant *Rhodococcus*, slowly growing in ethane-containing medium, but the amount of methanol accumulated in the recombinant strain was still low when methane was added. Presumably pMMO was not correctly assembled within the membrane of heterologous host to have complete function of oxidizing various alkanes. It seems that it may be hardly possible to reproduce pMMO in the form of recombinant whole protein complex with native activity in heterologous bacterial membrane.

It is a big question why pMMO is a membrane protein. Electrons may be well transferred to active site of pMMO through the transmembrane regions. Balasubramanian et al. [54] also assumed that increased solubility of methane in the lipid bilayer of membrane causes a high local concentration of methane, resulting in efficient substrate entry through the membrane. Another plausible reason is that methanol product can be easily released into periplasmic space where the next metabolic enzyme, methanol dehydrogenase is located. It looks likely that pMMO is a more rationally designed enzyme by nature than sMMO that is located in cytoplasm. The membrane-bound pMMO-mimic biocatalytic system could provide a novel optimal solution to the biological methane oxidation problem.

CONCLUSION

Methane, the primary component of natural/shale gas, is a useful and abundant natural resource, and the conversion of methane to methanol contributes to the economically viable utilization of natural/shale gas. Methanol is a very valuable liquid fuel and raw material for synthetic hydrocarbons and related products. The industrial production of methanol is currently based on a two-step process that is energy-intensive and environmentally unfriendly, requiring extreme operating conditions such as high pressure and high temperature, although the direct and selective oxidation of methane is attractive as a notable alternative. Clearly, the biological oxidation of methane to methanol, which is based on the activity of methane monooxygenase that is found in various methanotrophic bacteria, is more desirable as the reactions can be operated under mild conditions with a high selectivity, but the crucial aspect is the ability to improve the conversion rate and yield up to an industrially viable level. Additionally, maintaining the stable enzyme activity under reaction conditions is also important.

According to the metabolic pathways of methanotrophic bacteria, methanol is not the final product and only a precursor of formaldehyde that is used to synthesize various metabolites essential to survival of bacteria, and therefore the use of native bacteria seems not suitable for selective production of a sufficient amount of methanol. Metabolic engineering based on genome sequence information does not seem to open a great opportunity for overproduction of methanol, either. The engineering of methane monooxygenase based on understanding of the basic mechanisms of enzymatic methane oxidation would give a better chance to develop efficient biocatalysts. There are two types of methane monooxygenase: soluble cytoplasmic enzyme (sMMO) and membrane-bound particulate enzyme (pMMO). sMMO, consisting of three components (reductase, hydroxylase, and regulatory protein), has a dimeric structure, featuring an $(\alpha\beta\gamma)_2$ dimer architecture. The detailed mechanism of methane oxidation by sMMO was already discovered in detail, explaining that the di-iron site within hydroxlase is the active center for methane oxidation. Recent atomic-level information about the hydroxylase-regulatory protein complex makes it possible to delineate access pathways of methane to the active site of sMMO. Compared to sMMO, pMMO, a trimer (pmoA, pmoB, and pmoC) in an $\alpha_3\beta_3\gamma_3$ polypeptide arrangement is not so well understood that there has been a controversy regarding which one among the three metal centers in each $\alpha\beta\gamma$ protomer is a true active site. However, the controversy has been recently resolved by elucidating that pMMO is a copper enzyme with a di-copper active site located in the N-terminal soluble domain of pmoB subunit, although the role of the transmembrane regions is not yet clear and needs to be clarified in future.

On the basis of the fundamental and detailed information about enzymatic methane oxidation, the engineering/evolution/modification of MMO enzymes using various biological and chemical techniques may lead to an optimal way for the significant enhancement of catalytic activity, reaction stability, and/or solubility in organic solvents. Also, the technically and economically viable preparation of electron donors (reductants) is an important aspect. It seems that pMMO is a more rationally designed enzyme by nature than sMMO that is located in cytoplasm, based on the fact that electrons are well transferred through the transmembrane regions and that presumably increased solubility of methane in the lipid bilayer of membrane causes a high local concentration of methane, resulting in efficient substrate entry through the membrane. The development of membrane-bound pMMO-mimic biocatalyst could be an optimal way to reach the ultimate goal, i.e., technically and economically feasible and environmentally friendly oxidation of methane, for which multidisciplinary efforts from chemical engineering, protein engineering, and bioprocess research sectors should be systematically combined.

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