Biocatalytic Conversion of Methane to Methanol as a Key Step for Development of Methane-Based Biorefineries

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Methane is considered as a next-generation carbon feedstock owing to the vast reserves of natural and shale gas. Methane can be converted to methanol by various methods, which in turn can be used as a starting chemical for the production of value-added chemicals using existing chemical conversion processes. Methane monooxygenase is the key enzyme that catalyzes the addition of oxygen to methane. Methanotrophic bacteria can transform methane to methanol by inhibiting methanol dehydrogenase. In this paper, we review the recent progress made on the biocatalytic conversion of methane to methanol as a key step for methane-based refinery systems and discuss future prospects for this technology.

Keywords: Methane, methane monooxygenase, methanotrophs, methanol, natural gas, shale gas

Introduction

Methane is the primary component in natural and shale gas, comprising up to 90% of the total abundance. The world reserves of natural gas and shale gas have been estimated as being more than 6,614 [33] and 7,299 [26] trillion cubic feet, respectively. Thus, methane is considered as a next-generation carbon feedstock. In addition, methane constitutes more than 50% of the biogas generated from anaerobic digestion processes and landfill sites [16]. On the other hand, methane has attracted a great deal of attention from environmental scientists because it is the second most

abundant greenhouse gas after carbon dioxide and 21 times more harmful [51].

At present, methane has very limited use as a simple combustion fuel. Accordingly, there are significant needs to efficiently utilize methane from natural and shale gas and add value to methane. Indeed, compared with methane, methanol can be more readily used as a starting chemical, as well as a fuel in gasoline blending [48]. The energy density of methanol (15.8 MJ/l) is higher than that of methane (38.1×10^{-3} MJ/l). In addition, the market demand for methanol as a carbon feedstock is growing because it can be converted to value-added derivatives such as

methyl-*tert*-butyl ether and dimethyl ether [39]. The roles of various methanol-based chemical processes such as methanol-to-propylene and methanol-to-olefin conversion are also becoming significant [41].

Methane can be converted to methanol using indirect two-step methods. In this process, methane is first transformed to a syngas of CO and H₂ using a reforming process, which is followed by transformation to methanol using metal catalysts. However, the conversion of methane and water to carbon monoxide and hydrogen in this way is an energy-intensive process that requires high temperatures (>900°C) and costly equipment, accounting for approximately 60% of the total capital cost of the reaction process [15, 35, 46]. In addition, this reaction process has a low overall conversion rate of less than 25% and selectivity less than 70% [2, 32, 42]. Thus, the indirect conversion of methane to methanol is associated with a number of problems, including low energy efficiency and high capital cost [58].

Methanol can also be produced directly from methane by biological methods. Importantly, the bioconversion of methane to methanol is associated with low energy consumption, high conversion, high selectivity, and low capital costs compared with chemical methods, since it is carried out by methane monooxygenase (MMO) enzyme or methanotrophic cells under mild conditions [8]. MMO activates the stable C-H bond of methane under ambient conditions, whereas methanotrophs express MMO for methane oxidation and utilize methane as their sole carbon and energy source under aerobic conditions. However, methanotrophs grow slowly, and thus the production of methanol from whole cells of methanotrophs is also low [11]. Because methanotrophs utilize methanol as both carbon and energy sources, one disadvantage of cell-based methane-to-methanol conversion is that the methanol is further metabolized to formaldehyde, formate, and carbon dioxide by methanol dehydrogenase, formaldehyde dehydrogenase, and formate dehydrogenase, respectively [59]. Thus, a high volumetric production of methanol by methanotrophs is currently difficult to achieve.

Methane-to-methanol conversion is attracting considerable attention owing to the abundant reserves of methane and the potential for utilization of methane as an alternative chemical feedstock for the production of value-added chemicals. Bioconversion may be a good choice for methane-to-methanol conversion, because bioconversion can be accomplished in an energy-efficient and environmentally benign manner. Herein, we review the recent progress on the biocatalytic conversion of methane to methanol using MMO and methanotrophs as biocatalysts and discuss the biotechnological potential of methane-to-methanol bioconversion.

Methane-to-Methanol Bioconversion by Methane Monooxygenase

Methane is chemically very stable owing to the presence of C-H bonds [5, 18, 50], which require 438.8 kJ/mol to break [40]. MMO efficiently converts methane to methanol in biological systems [37] by splitting molecular oxygen and incorporating one atom of oxygen into methane. There are two classes of MMOs, namely, soluble MMO (sMMO) and membrane-associated MMO, the latter of which is also referred to as particulate MMO (pMMO). Although both sMMO and pMMO catalyze methane oxidation, they exhibit significantly different catalytic characteristics, including different enzyme stabilities, specific activities,

Fig. 1. Structural overview of *Methylococcus capsulatus* (Bath) soluble methane monooxygenase consisting of three subunits (A) and the homodimeric structure of hydroxylase containing a di-iron center (B) (modified from Friedle *et al.* [13]).

and susceptibilities to inhibitors. For example, in terms of reducing power for the reaction, sMMO has a strict requirement for nicotinamide adenine dinucleotide (NADH), whereas pMMO can utilize the reducing power generated by formaldehyde dehydrogenase (FaldH) [52, 60]. The active sites of sMMO and pMMO also contain different metal ions, namely, iron and copper, respectively. pMMO is expressed under a high copper concentration [4, 30, 38, 49].

The catalytic mechanism of sMMO isolated from *Methylococcus capsulatus* (Bath) has been studied extensively. sMMO consists of three subunits with hydroxylase (MMOH, 251 kDa), reductase (MMOR, 38.6 kDa), and regulatory functions (MMOB, 15.9 kDa) (Fig. 1A) [29]. On the other hand, MMOH is a homodimeric protein consisting of two sets of α , β , and γ subunits containing a di-iron site, which is the primary domain responsible for catalyzing the conversion of methane with oxygen to methanol (Fig. 1B). MMOR contains a bound flavin adenine dinucleotide (FAD) and a [2Fe-2S]-ferredoxin (Fd) cofactor, which play key roles in the activation of MMOH by transferring reducing power to MMOH using NADH. MMOB is known to regulate the structure and reactivity of the non-heme di-iron site of MMOH [13, 45, 47]. In order to activate molecular oxygen, the di-iron center in MMOH undergoes cyclical changes, such as with intermediate P and intermediate Q and various states of diferrrous, peroxy, diferryl, and diferric iron (Fig. 2). With respect to diferrrous MMOH in reduced form, the di-iron centers react with molecular oxygen to form a peroxide species, intermediate

P. Intermediate P can then be converted to intermediate Q, which contains an Fe^{IV} center. Intermediate Q in turn abstracts hydrogen from methane to form QH and a free methyl radical, suggesting a radical mechanism. Methanol is eliminated immediately after transferring the OH group to the free radical, resulting in regeneration of the catalyst. In order to convert methane to methanol by sMMO, reducing power in the form of NADH is required to activate molecular oxygen [31].

Most methanotrophs constitutively express pMMO, which is the membrane-integrated form of MMO that is expressed in the presence of high concentrations of copper. As described above, pMMO comprises three subunits;

Fig. 2. Proposed catalytic mechanism of soluble methane monooxygenase (modified from Friedle *et al.* [13]).

Fig. 3. Structural overview of the particulate methane monooxygenase (pMMO) (A) and the tri-copper cluster and the di-iron cluster in the intermembrane pmoA and pmoC subunits of the enzyme (B) (modified from Bollinger [6] and Culpepper and Rosenzweig [9]).

Fig. 4. Proposed catalytic mechanism of particulate methane monooxygenase (modified from Balasubramanian and Rosenzweig [4]).

namely, pmoA, pmoB, and pmoC. pMMO has a trimeric structure in an $\alpha 3\beta 3\gamma 3$ polypeptide arrangement (Fig. 3) [6, 9, 30]. The pmoA and pmoC subunits exist in the membrane in a helical structure. In contrast, pmoB has two transmembrane helices and a long loop region linked by a β -barrel motif. This soluble domain forms a hole for substrate entry and product exit and plays a key role in catalysis [17]. Structural analysis of pMMO from *M. capsulatus* has revealed that the active site comprises a mononuclear copper site (coordinated by His 48 and His 72), a dinuclear copper site (coordinated by His 33, His 137, and His 139), and zinc to facilitate various oxidation states to activate O_2 [9]. Sequential formation of Cu(I)Cu(I), peroxy Cu(II)Cu(II), *bis*(μ -oxo) Cu(III)Cu(III), and *bis*(μ -oxo) Cu(III)Cu(II) has been proposed for molecular oxygen activation by pMMO (Fig. 4) [61].

Despite significant progress on the analysis of the structure and function of sMMO and pMMO, biotechnological application of isolated MMO enzymes remains limited owing to an inability to express functional protein in *Escherichia coli*. Furthermore, the isolation of significant

quantities of functional sMMO and pMMO from methanotrophs is also somewhat difficult. However, in order to achieve enzyme-based methane-to-methanol bioconversion on a biotechnological scale, the functional expression and isolation problems need to be overcome, in addition to the development of an appropriate *in vitro* reducing power regeneration system.

Methane-to-Methanol Bioconversion by Methanotrophs

Methanotrophs are bacteria that utilize methane as their sole source of carbon for growth and are capable of activating stable C-H bonds using MMO under ambient conditions [40]. Methanotrophs have recently attracted a great deal of attention from the field of biodegradation, specifically with regard to a wide range of priority pollutants, including trichloroethylene, through co-metabolic reactions catalyzed by MMO [20, 27]. In general, methanotrophs can be classified into three groups based on physiology (Fig. 5). Gammaproteobacterial methanotrophs are Type I

Fig. 5. Classification of methanotrophs based on physiology (modified from the Methanotrophs Consortium website, 2014).

methanotrophs and utilize the ribulose monophosphate (RuMP) cycle for carbon assimilation. This group comprises the genera *Methylococcus* and includes *M. capsulatus* and *Methylomonas*. The RuMP cycle allows for the synthesis of key intermediates for central metabolic routes from formaldehyde (Fig. 6). In this system, carbon is fixed into the cell biomass *via* aldol condensation of formaldehyde with D-ribulose-5-phosphate to form 3-hexulose 6-phosphate. Type II methanotrophs are Alphaproteobacteria and comprise the *Methylosinus* genera including *M. trichosporium*. Type II methanotrophs use the serine cycle for carbon assimilation and have a functional TCA cycle. Specifically, 3-phosphoglycerate is synthesized from formaldehyde and CO₂ *via* the serine cycle. A novel Type III class of thermoacidophilic aerobic methanotrophs called Verrucomicrobia was recently identified and was also shown to assimilate CO₂ using the Calvin Benson Bassham cycle [12, 43]. Importantly, *Methyloacidiphilum fumariolicum* has the potential to be cultivated using a mixture of methane and CO₂ to improve cell growth and product yield [23].

In terms of methane conversion to methanol, many

methanotrophs, including *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* (Bath), have been employed. Methanotrophs can convert methane to carbon dioxide through four successive metabolic steps, consisting of methane to methanol, methanol to formaldehyde, formaldehyde to formate, and finally formate to carbon dioxide (Fig. 5) [7, 10, 56, 57]. The classical periplasmic MxaF-type methanol dehydrogenase is a pyrroloquinoline quinone (PQQ)-dependent enzyme composed of an $\alpha_2\beta_2$ tetramer [1]. Methanol dehydrogenase (MDH)-catalyzed oxidation of methanol to formaldehyde is coupled to a periplasmic mono-*c*-type cytochrome and cytochrome *c_L* in obligate aerobic methanotrophs such as *M. capsulatus* [57]. In order to accumulate methanol, MDH should be inhibited. Therefore, the functional expression of highly active MMO and the accumulation of methanol by inhibition of MDH are essential features of methanotrophic conversion of methane with high methanol productivity under aerobic conditions [59].

The selectivity and conversion of methane to methanol by methanotrophs are very high owing to the excellent

Fig. 6. Simplified methane metabolic pathways of the ribulose monophosphate (RuMP) cycle and serine cycle in methanotrophs. Major enzymes and metabolic intermediates are presented in the metabolic pathways (modified from Chistoserdova and Lidstrom [7]).

performance of sMMO and pMMO. The expression pattern and abundance of MMO are highly dependent on the copper concentration of the medium. When copper is limited, sMMO is present in the cytosol, whereas pMMO is primarily expressed in the presence of high levels of copper [17, 34, 44, 49]. In addition, sMMO and pMMO differ from each other with respect to substrate and inhibitor specificities, enzyme stability, relative activities, and reducing power requirements and generation. pMMO has a higher affinity for methane compared with sMMO [31]. In terms of reducing power utilization, sMMO utilizes NADH, whereas pMMO uses electrons from ubiquinol-based electron transfer systems associated with the FdH-catalyzed reaction of formaldehyde to formate [10]. For increased methanol productivity in sMMO-catalyzed methane oxidation, exogenous NADH should be supplied because one mole of NADH is stoichiometrically required for the oxidation of one mole of methane to methanol. In methanotroph-mediated metabolism, NADH is generated when FdH and FDH convert formaldehyde to formate and formate to carbon dioxide, respectively. Thus, when the activity of MDH is fully inhibited, NADH cannot be generated. Formate is commonly supplied as a co-substrate for methanotrophic methanol production with MDH inhibition, because one mole of formate oxidation by formate dehydrogenase generates one mole of NADH from NAD [22, 62].

Methanotrophic methane-to-methanol conversion on the biotechnological scale has been investigated primarily using *M. trichosporium* OB3b, which can be cultured on methane in Higgins nitrate mineral salt medium containing 5 μM Cu^{2+} to express pMMO [36, 55]. However, in order to prevent further oxidation of methanol, the MDH activity must be efficiently inhibited. Various MDH inhibitors, including metal chelating agents such as EDTA, cyclopropane, cyclopropanol, dithiothreitol, phenylhydrazine, NaCl, iodoacetate, MgCl_2 , and high concentrations of phosphate, have been employed to prevent methanol conversion to formaldehyde. Indeed, the presence of chelating agents such as EDTA in media significantly inhibits the activities of methanol oxidase and MDH [59]. When cyclopropanol is used for the irreversible inhibition of methanol dehydrogenase, *M. trichosporium* OB3b could produce methanol with a production efficiency of 61% and a methanol concentration of 152 mmol/g dry cell for 100 h [54]. Furuto *et al.* [14] also employed cyclopropanol to inhibit methanol dehydrogenase activity, obtaining 6 mM methanol using a semi-continuous process.

NaCl (200 mM) has also been successfully employed for

MDH inhibition in *M. trichosporium* OB3b to achieve methane-to-methanol conversion, resulting in 7.7 mM methanol from 0.6 mg dry cells/ml at 25°C in 12.9 mM phosphate buffer (pH 7) containing 20 mM sodium formate with a supply of methane/air gas (1:4 (v/v)) for 36 h [28]. However, when NaCl was used for MDH inhibition in *M. trichosporium* OB3b, the activities of both pMMO and MDH were inhibited, although the extent of enzyme inhibition for MDH was much greater than that of pMMO, thereby allowing for methanol accumulation.

A novel compulsory circulation diffusion system was developed for the cultivation of *M. trichosporium* OB3b under optimal conditions (5 μM CuSO_4 , pH 7.0, 30°C) with a supply of methane/air (1:1 (v/v)) prepared in a tightly sealed gas reservoir [25]. A cell concentration of 0.6 mg dry cells/ml in 10 mM phosphate buffer (pH 7.0) (working volume of 100 ml in a 500 ml flask) was used for methane-to-methanol conversion in batch and repeated-batch modes. In the presence of 100 mM NaCl and 1 mM EDTA as MDH inhibitors and 20 mM sodium formate supplied for reducing power regeneration, 13.2 mM methanol was produced in the batch reaction at 25°C for 12 h. In the case of the repeated-batch mode, a productivity of 2.17 μmol methanol/h/mg cell was obtained for 24 h with three 8 h cycles [24]. Recently, more than 1.12 g/l methanol was accumulated with 60% conversion using 17 g dry cell/l of *M. trichosporium* OB3b in the presence of 400 mmol/l phosphate buffer, 10 mmol/l MgCl_2 , and 20 mmol/l sodium formate [11]. In addition, 0.95 g/l methanol accumulated in a membrane-aerated reactor when methane and oxygen were introduced from two separate sources through dense silicone tubing.

In addition to methanotrophs, ammonia-oxidizing bacteria such as *Nitrosomonas europaea* and *Nitrosococcus oceanii* have been shown to convert methane to methanol using ammonia monooxygenase (AMO). Although the substrate for AMO is ammonia, methane can be selectively and partially oxidized to methanol owing to the broad substrate specificity of AMO [3, 19, 21]. Similar to methanotrophs, the growth rate of ammonia-oxidizing bacteria is slow, and thus volumetric culture productivity is also low. One advantage of ammonia-oxidizing bacteria for methanol production is that it is difficult to further metabolize methanol owing to the absence of relevant enzymes, resulting in an accumulation of methanol in the medium. In order to maintain the metabolic activity of AMO, ammonia needs to be supplied as a growth substrate. Recently, mixed nitrifying enrichment cultures were used for methane-to-methanol conversion. Using a fed-batch reactor, 59.9 mg methanol (expressed in

COD)/l was produced for 7 h with a maximum specific rate of 0.82 mg methanol COD/mg biomass COD. Thus, partial oxidation of methane to methanol by AOB may be an alternative to methanotroph-mediated conversion [53].

Concluding Remarks and Future Prospects

To date, unsuccessful heterologous MMO expression in *E. coli* is the primary obstruction for the industrial application of methane-to-methanol conversion. Thus, the development of a recombinant MMO enzyme is a prerequisite to enzyme-based bioconversion of methane to methanol. A higher productivity of methane to methanol will require the molecular engineering of MMO for enhanced activity, which will become more feasible as our understanding of the structure and catalytic mechanism of MMO improves. Although MMO is an efficient enzyme for methane oxidation under mild reaction conditions, regeneration of the reducing power such as NADH for efficient utilization of molecular oxygen for the monooxidation of methane is a barrier to the commercialization of MMO-catalyzing methane oxidation reactions. Regeneration of the reducing agent using an electrochemical method in the presence of an enzyme biocatalyst can be a solution. The molecular engineering of other monooxygenases such as P450 to accept methane as a substrate is yet another possible solution for efficient methane conversion to methanol, since P450 monooxygenase can be heterologously expressed in *E. coli*.

Metabolically engineered methanotrophs for enhanced methane-to-methanol productivity have not yet been constructed since genetic tools for methanotroph engineering are limited. At present, wild-type methanotrophic cell-based methane-to-methanol conversion is limited by slow growth rates and poor specific and volumetric productivities in the range of $\mu\text{mol}/\text{mg}\cdot\text{h}$. To achieve higher rates of methane-to-methanol conversion, efficient methods for methanol accumulation and reducing power regeneration need to be developed by redesigning the metabolic pathway using metabolic engineering and synthetic biology approaches. Indeed, the reconstruction of the methanotroph metabolic network will likely be a key approach for the development of a microbial cell factory to produce a variety of chemicals from methane. In addition, the circumvention of product inhibition will be required in cases where methanol is accumulated at high concentrations. The screening of novel methanotrophs using omics-based approaches is another possible tool for identifying higher methane utilizers. Lastly, methane mass transfer needs to be enhanced for

higher productivity.

The market demand for methanol is continuously increasing owing to its versatility in chemical synthesis. Thus, methane-based methanol can be considered as a drop-in item that can be readily commercialized upon development of a cost-effective biocatalytic method of methane-to-methanol conversion in the near future. The cost of methanol production from methane will be competitive owing to methane abundance, especially with the recent boom in shale gas production. In addition, methanol transformation using biocatalytic conversion methods may be especially applicable to countries with small-scale gas fields and poor infrastructure, owing to the low capital investment of the bioprocess for methane-to-methanol conversion compared with existing indirect chemical methods and the flexibility of biocatalytic methane-to-methanol processes.

In conclusion, the development of efficient biocatalytic methane-to-methanol conversion methods will play a key role in the commercialization of methane reforming, and the production of a variety of chemicals from methanol has the potential to replace the petroleum-based production of chemicals. The commercial success of biocatalytic methane-to-methanol conversion may be a starting point for a paradigm shift from petroleum-based chemical refineries to gas-based refineries.

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