RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Journal Name

RSCPublishing

COMMUNICATION

Methane hydroxylation using light energy by the combination of thylakoid and methane monooxygenase

Cite this: DOI: 10.1039/x0xx00000x

Hidehiro Ito,‡[a] Fumiya Mori,‡[b] Kenji Tabata,[c] Ichiro Okura[b] and Toshiaki Kamachi*[b]

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

Photoinduced methane hydroxylation system was established by the combination of photosynthetic system of thylakoid and methane monooxygenase (MMO) from *Methylosinus trichosporium* OB3b. By using photosynthetic system, the electrons needed for direct oxidation of methane to methanol can be obtained from water under light irradiation.

Methane is the primary component of natural gas, which is a promising alternative fuel source because "shale gas revolution" has led to major increases in reserves of natural gas. Methane can be also produced from carbon dioxide by anaerobic process called methanogenesis using methanogen.[1,2] Methane is usually liquefied for ease of storage or transport, but liquefaction of natural gas is uneconomic not only because it requires gas to be cooled to very low temperatures but also transportation or storage facilities needs specially designed refrigerated and insulated tanks. Therefore, the conversion of methane to methanol is desired for the usage of natural gas. Olah *et. al.* said methanol is an excellent fuel in its own right and it can also be blended with gasoline.[3] Furthermore, they suggested that methanol had the possibility if an alternative energy carrier to diminishing oil–the so-called "methanol economy". However, selective oxydation of methane to methanol is extremely difficult chemistry because the C-H bond in methane has one of the highest bond energy (104 kcal mol^{-1}) amongst organic substrates. Recently, several groups have reported the direct oxidation of methane to methanol using transition metal catalyst[4-8] and metalloenzymes,[9,10] but the development of efficient catalyst for direct conversion of methane to methanol is still one of the most challenging subjects in the catalytic chemistry.

In nature, on the other hand, the hydroxylation of methane is catalyzed by an important class of enzymes known as methane monooxygenases (MMO), which are unique in their capability to mediate the facile conversion of methane to methanol under ambient conditions.[11] Therefore, many researchers have studied about MMO's structure, active site and mechanism of the oxidation of methane to methanol until now.[12-14] The oxidation of methane to methanol by MMO requires two electrons and molecular oxygen as shown in Scheme 1, so it is important to select an appropriate electron source in the methane hydroxylation, especially for industrial application.

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

Scheme 1 Synthesis of methanol from methane by methane monooxygenase.

In this work, we describe the construction of a photoinduced methane hydroxylation system by the combination of photosynthetic system and methane monooxygenase (Fig. 1). By using photosynthetic system, the electrons needed for direct oxidation of methane to methanol can be obtained from water under light irradiation. Here, we used thylakoid from spinach for water oxidation to supply electrons needed for selective oxidation of methane catalyzed by MMO. Membrane fraction from *Methylosinus trichosporium* OB3b, which includes a particle methane monooxygenase (pMMO), was used for the catalyst of methane oxidation.

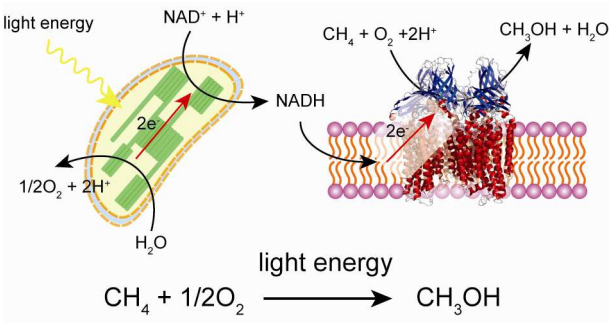

Fig. 1 Schematic diagram of photoinduced methane oxidation system using methane monooxygenase by the combination of photosynthetic system.

Isolation of thylakoid membrane from spinach was performed according to the previous methods (detail is shown in the Supporting Information).[15,16] The photoreduction of $NAD(P)^+$ by thylakoid membrane was carried out by the irradiation of visible light to the mixture containing thylakoid (0.3 mg-chl/mL) and 2.0 mM $NADP^+$ or NAD^+ in 50 mM Tricine buffer (0.4 M Sucrose, 10 mM NaCl, 2 mM $MgCl_2$ and 50 mM Tricine adjusted pH 8.0 with KOH aq.) using MME250 high power metal halide light source (250W, Moritex Japan) at a 10% output power through 470 nm cut off filter (Y-47). The photoreduction of $NAD(P)^+$ to NAD(P)H was measured by the absorbance at 340 nm in the supernatant of reaction mixture.[17,18] Time dependence of NAD(P)H production suggested that the activity of the thylakoid was maintained over 1 hour (Fig. S1) and $NADP^+$ was better electron acceptor for thylakoid membrane than NAD^+.

The membrane fraction from *M. trichosporium* OB3b was isolated by the sonication of the bacterial cells followed by the ultracentrifugation according to previously reported procedures.[19] Methane monooxygenase activity was measured using methane or propylene as a substrate as follows. The reaction mixture containing 2.0 mg-protein/mL membrane fraction from *M. trichosporium* OB3b and 2.0 mM NADH in 50 mM Tricine buffer (pH 8.0) was prepared in the vial sealed with an open-top cap with Teflon septa. The reaction was initiated by the injection of substrate into the reaction vial with a gas-tight syringe and was carried out in thermostatic water bath at 30 °C. The amount of oxidation products was measured by FID-gas chromatograph (HITACHI 263-30) attached with a Sorbitol 25%-Gasport B 60/80 in a glass column. Results of MMO activity of membrane fraction from *M. trichosporium* OB3b are shown in Fig. 2. These results showed the limited operational stability of only 20-30 min of the pMMO in membrane fraction from *M. trichosporium* OB3b as opposed to 1 hour for the thylakoid. Previous report showed that MMO activity of membrane fraction from *Methyloccus capsulatus* using NADPH was not observed, while the cofactor specificity of MMO from *M. trichosporium* OB3b has not been reported.[20] The pMMO activity of membrane fraction from *M. trichosporium* OB3b is higher when NADH was used as an electron donor compared to NADPH (date not shown). This result suggested that the NADH-quinine oxidoreductase, which is electron transfer protein in the membrane fraction from *M. trichosporium* OB3b, shows selectivity for NADH over NADPH.

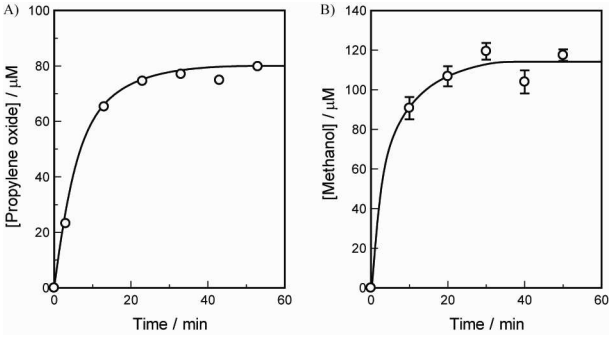

Fig. 2 Time dependence of the formation of oxidation products with membrane fraction from *M. trichosporium* OB3b using NADH. A) Propylene oxidation, B) methane oxidation.

Photoinduced propylene oxidation was carried out by the irradiation of light to the mixture containing chlorophyll from spinach and pMMO containing membrane fraction from *M. trichosporium* OB3b in the presence of $NADP^+$ and NAD^+ as an electron carrier. As $NADP^+$ is the terminal electron accepter of photosynthetic reaction, NADPH reduced by photosystem is supposed to be transformed into NADH by $NAD(P)^+$ transhydrogenase activity using ferredoxin-$NADP^+$ reductase (FNR) from thylakoid. The reason for the usage of propylene instead of methane as substrate is that our photoinduced methane hydroxylation system contains various enzymes both from spinach and *M. trichosporium* OB3b so that methanol, oxidation products of methane, can be further oxidized. The mixture of thylakoid (0.3 mg-chl/mL), membrane fraction from *M. trichosporium* OB3b (2.0 mg-protein/mL) and 2.0 mM $NADP^+$ and 2.0 mM NAD^+ in the reaction vial sealed with a Teflon-septa was prepared under air atmosphere. After injection of 0.3 mL propylene to the reaction vial using gas-tight syringe, the reaction was initiated by the irradiation of visible light at 30 °C. Fig. 3A showed the time-dependent change in the production of propylene oxide by this system. As light irradiation time increased, production of propylene oxide increased and approached the maximum value. However, in the reaction mixture containing 2.0 mM $NADP^+$, the production of propylene oxide was very low. The oxidation of propylene increased with decreasing the concentration of $NADP^+$ in the reaction mixture and highest activity was observed without $NADP^+$. The pMMO activity increased with increasing the concentration of NAD^+ in the reaction mixture without $NADP^+$ (Fig. 3B). These results suggested that $NAD(P)^+$ transhydrogenase activity from FNR didn't work well in this system, indicating that $NADP^+$ has an inhibitory effect on the overall reaction system. Therefore, we decided to use only NAD^+ as an electron carrier in this system.

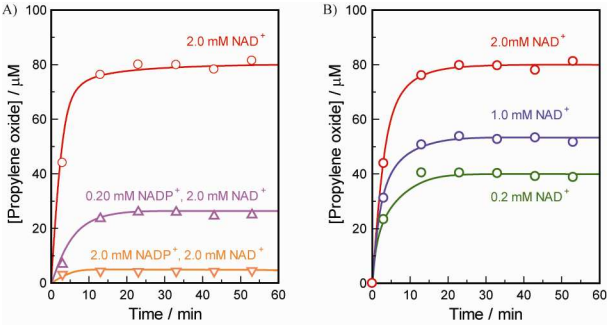

Fig. 3 Effect of the concentration of electron carriers in the reaction mixture containing 0.3 mg-chl/mL thylakoid and 2.0 mg-protein/mL membrane fraction of *M. trichosporium* OB3b with light irradiation.

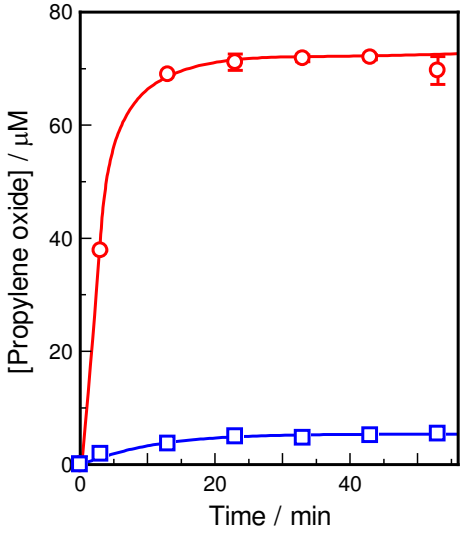

Fig. 4 Time dependence of propylene oxide concentration in the reaction mixture containing 2.0 mM NAD^+, the thylakoid, membrane fraction of *M. trichosporium* OB3b and 0.3 mL propylene with (red, open circle) or without (blue, open square) light irradiation.

Propylene oxide was almost not observed in this system without light irradiation (Fig. 4). Propylene oxide obtained by the oxidation of propylene by MMO was not further metabolized by the enzymes present in the reaction mixture. These results indicate that the photoreduction of NAD^+ occurs by the irradiation of light to the thylakoid membrane and produced NADH is used for the oxidation of propylene by pMMO. Amount and velocity of propylene oxide formation by the combination of thylakoid membrane and pMMO under irradiation of light was almost same as the pMMO activity without irradiation of light using NADH as an electron donor with pMMO containing membrane fraction from *M. tricosporium* OB3b alone. This result indicates that oxidation of propylene by pMMO is the rate limiting step in the photoinduced propylene oxidation system (Supporting Information, Fig. S2). Propylene oxide is a chiral compound. Stereoselectivity of propylene oxidation using methylotroph has been reported to be very low (*R*:*S* = 57:43).[21,22] But this report used bacterial cell and they did not mentioned the kind of methane monooxygenase (membrane bound or soluble). So stereoselecctivity of the membrane bound MMO should be studied further.

Finally, selective oxidation of methane to methanol was carried out using a same system mentioned above. Methane was injected in the reaction vial in place of propylene. Methane oxidation reaction was initiated by the irradiation of visible light at 30 °C. Fig. 5 showed the time-dependent change in the production of methanol by this system. By the irradiation of light to the reaction mixture, the amount of methanol was increased. The amount of methanol obtained in the dark condition was apparently lower than that obtained under irradiation of light (Supporting Information). These results indicate that selective oxidation of methane to methanol can be achieved by using electrons obtained from water oxidation by chlorophyll under irradiation of light.

Conclusions

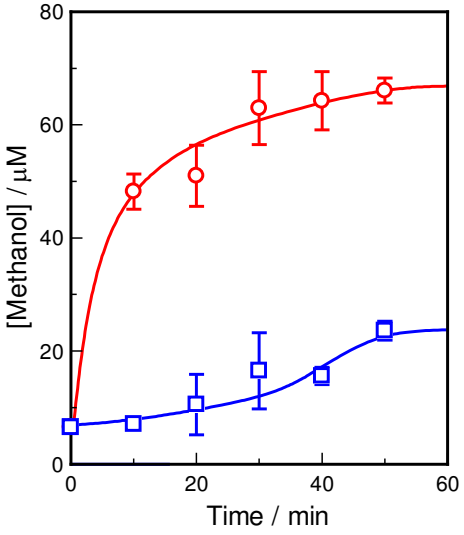

Fig. 5 Time dependence of methanol concentration in the reaction mixture containing 2.0 mM NAD^+, the thylakoid, membrane fraction from *M. trichosporium* OB3b and 0.3 mL methane with (red, open circle) or without (blue, open square)

In conclusion, we achieved the hydroxylation of methane using light energy by the combination of thylakoid and membrane fraction of *M. trichosporium* OB3b. In our system, pMMO can activate oxygen using NADH, which is a product of water oxidation under irradiation of light, and can catalyze the selective oxidation of substrate such as methane or propylene. This result indicates that methane can be oxidized using water as a reductant under irradiation of light. However, low stability of the pMMO in the membrane fraction from *M. trichosporium* OB3b limits the application of our system for long term usage. So we are now trying to stabilize pMMO to increase the conversion of methane to methanol.

Notes and references

[a] Education Academy of Computational Life Sciences, Tokyo Institute of Technology, 4259, Nagatsuta, Midoriku, Yokohama, 226-8501, Japan.. E-mail: hito@bio.titech.ac.jp; Fax: +81 45 924 5778; Tel: +81 45 924 5753

[b] Department of Bioengineering, Tokyo Institute of Technology, 4259, Nagatsuta, Midoriku, Yokohama, 226-8501, Japan. E-mail: tkamachi@bio.titech.ac.jp; Fax: +81 45 924 5778; Tel: +81 45 924 5752

[c] Frontier Research Center, Tokyo Institute of Technology, 4259, Nagatsuta, Midoriku, Yokohama, 226-8501, Japan. E-mail: ktabata@bio.titech.ac.jp; Tel: +81 45 924 5126

† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/c000000x/

‡ These authors contributed equally.

Acknowledgment

This work was partially supported by Grant-in-Aid for Scientific Research (C) number 24550183 from Japan Society for the Promotion of Science (JSPS).

1. J. G. Ferry, FEMS Microbiology Reviews, 1999, 23, 13-38.
2. R. N. Austin and J. T. Groves, Metallomics, 2011, 3, 775-787.
3. G. A. Olah, Angewandte Chemie International Edition, 2005, 44, 2636-2639.
4. B. A. Arndtsen, R. G. Bergman, T. A. Mobley and T. H. Peterson, Accounts of Chemical Research, 1995, 28, 154-162.
5. R. A. Periana, D. J. Taube, S. Gamble, H. Taube, T. Satoh and H. Fujii, Science, 1998, 280, 560-564.
6. L. Que and W. B. Tolman, Nature, 2008, 455, 333-340.
7. A. Caballero and P. J. Perez, Chemical Society Reviews, 2013.
8. G. B. Shul'pin, Dalton Transactions, 2013, 42, 12794-12818.
9. M. M. Chen, P. S. Coelho and F. H. Arnold, Advanced Synthesis & Catalysis, 2012, 354, 964-968.
10. C. Li and S. Shaik, RSC Advances, 2013, 3, 2995-3005.
11. M. Takeguchi, T. Furuto, D. Sugimori and I. Okura, Appl Biochem Biotechnol, 1997, 68, 143-152.
12. A. S. Hakemian and A. C. Rosenzweig, Annu Rev Biochem, 2007, 76, 223-241.
13. R. Balasubramanian, S. M. Smith, S. Rawat, L. A. Yatsunyk, T. L. Stemmler and A. C. Rosenzweig, Nature, 2010, 465, 115-119.
14. C. T. Armstrong, D. W. Watkins and J. L. R. Anderson, Dalton Transactions, 2013, 42, 3136-3150.
15. E. Andreasson, P. Svensson, C. Weibull and P.-Å. Albertsson, Biochimica et Biophysica Acta (BBA) - Bioenergetics, 1988, 936, 339-350.
16. T. Kieselbach, Hagman, B. Andersson and W. P. Schroder, J Biol Chem, 1998, 273, 6710-6716.
17. K. Jensen, P. E. Jensen and B. L. Møller, ACS Chemical Biology, 2011, 6, 533-539.
18. K. Jensen, J. Johnston, P. O. Montellano and B. Møller, Biotechnology Letters, 2012, 34, 239-245.
19. A. Miyaji, Methods Enzymol, 2011, 495, 211-225.
20. D. W. Ribbons and J. L. Michalover, FEBS Lett, 1970, 11, 41-44.
21. M. Ono, I. Okura, J. Mol. Catal., 1990, 61, 113-122.
22. S. Aono, M. Ono, I. Okura, J. Mol. Catal., 1989, 49, L65-L67

We construct photoinduced methane hydroxylation system by the combination of thylakoid and methane monooxygenase from *Methylosinus trichosporium* OB3b.