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Properties of the membranes containing the particulate methane monooxygenase from *Methylosinus trichosporium* OB3b

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The particulate methane monooxygenase (pMMO) from *Methylosinus trichosporium* OB3b was partially purified and characterized by measuring the effects of reducing agents and additives, and the stability of pMMO was studied. Duroquinol was a suitable reducing agent, and pMMO was stabilized by bovine serum albumin (BSA). Among the additivies, the copper (II) ion stimulated pMMO at low concentration and inhibited at high concentration. The optimum conditions for pMMO activity were as follows: 45°C, pH 6.5 and 55 mM 3-morpholinopropanesulfonic acid (MOPS) buffer, and the rate of propene epoxide formation was 13.6 nmol min^{-1} mg^{-1} protein. ESR spectra indicate that the copper cluster in the membrane fraction is reduced by duroquinol and oxidized by dioxygen. The result suggests that the copper cluster is contained in the active site of pMMO.

Keywords: copper, *Methylosinus trichosporium* OB3b, particulate methane monooxygenase

Introduction

Methane monooxygenase (MMO) in methanotrophs catalyzes the hydroxylation of methane to methanol according to the following equation:

$$CH_4 + O_2 + 2e^- + 2H^+ \rightarrow CH_3OH + H_2O$$

The formation of a soluble or membrane-bound MMO strongly depends on the copper (II) concentration during the growth of *Methylosinus trichosporium* OB3b (Scott *et al.* 1991). At low copper-to-biomass ratios, the enzyme activity is in the soluble fraction and the enzyme is referred as the soluble MMO (sMMO). At higher copper-to-biomass ratios, methane hydroxylation is mainly in the membrane fraction and is catalyzed by the membrane-bound or particulate MMO (pMMO). Though sMMO has been purified and studied extensively both at the biochemical and the genetic levels, there are few studies concerning pMMO, because of its instability. In this paper we describe the partial purification and characterization of pMMO in the membrane fractions from *Methylosinus trichosporium* OB3b.

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Materials and methods

Materials

All the chemicals used were of the highest grade available and were used without further purification. Methane and propene were purchased from Fujibussan Co. (Tokyo, Japan). Bovine serum albumin (BSA), ovalbumin, myxothiazol, duroquinone (tetramethyl-*p*-benzoquinone), decyl-plastoquinone and DNase I (from bovine pancreas) were obtained from Sigma-Aldrich Japan K.K. (Tokyo, Japan). Phenylmethylsulfonyl fluoride (PMSF), 2,3-dimethylquinone, trimethylquinone (1,4-dihydroxy-2,3,5-trimethylbenzene) and 3-morpholinopropanesulfonic acid (MOPS) were obtained from Wako Pure Chemical Industries (Tokyo, Japan). Coenzyme Q_0 (2,3-miethoxy-5-methyl-1,4-benzoquinone) was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). β-nicotinamide adenine dinucleotide and its reduced form (NADH) were obtained from Boehringer Mannheim (Germany). Ethanol in NADH was removed by evaporation at room

temperature before use. Menaquinone (2-methyl-1,4-naphthoquinone) and the other chemicals were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan).

Culture of Methylosinus trichosporium OB3b

M. trichosporium OB3b was kindly provided by Professor J.D. Lipscomb of the University of Minnesota and was cultivated as described by Fox *et al.* (1990). Cultures were maintained on 3.5% agar plates with 1.25 μM $CuSO_4$. Large-scale growth was started in 200-ml baffle-walled shaking flasks containing 27 ml medium with 5 μM $CuSO_4$ and a 20% methane in 80% air atmosphere. The cultures were shaken at 30°C for approximately four days. Each culture was then transferred to a 500-ml baffle-walled shaking flask containing 100 ml medium with 10 μM $CuSO_4$ and maintained under the same conditions for two days. After this period, the cultures (260–390 ml total) were used to inoculate 2.3 l of medium containing 10–20 μM $CuSO_4$ in a three-liter fermenter (MBF-500M, EYELA, Tokyo, Japan). The fermenter was maintained at 30°C with continuous stirring, and 50% methane and 50% oxygen were introduced. After five to seven days the cells were harvested in logarithmic phase or initial stationary phase by centrifugation at $6800 \times g$ for 10 min at 4°C with a typical yield of *c.* 4 g wet-cell per liter. The bacterial pellet was washed with 25 mM MOPS buffer (pH 7.0) and resuspended in the 25 mM MOPS buffer (pH 7.0). The resuspended cells were rapidly frozen with liquid N_2 and stored at –80°C.

Isolation of membranes from M. trichosporium *OB3b*

The buffer used in isolation procedures was deoxygenated by purging nitrogen gas. Frozen cells were thawed at room temperature and diluted with 25 mM MOPS buffer (pH 7.0). the cells were broken by sonication (UD-201, TOMY, Tokyo, Japan) at 4°C under anaerobic conditions. Just prior to breaking the cells the suspension was supplemented with 300 μM $CuSO_4$ and with DNase I at a concentration of 10 μg ml^{-1} suspension. During breaking of the cells the suspension was supplemented with 1 mM PMSF in acetone. The broken cells were centrifuged at $27\,720 \times g$ for 10 min at 4°C to remove cell debris and unbroken cells. The supernatant, containing membrane, was then centrifuged at $143\,000 \times g$ for 90 min at 4°C. The supernatant was discarded, and the membranes were resuspended by using homogenizer in 25 mM MOPS buffer (pH 7.0) containing 1 M KCl, and centrifuged at $143\,000 \times g$ for 90 min at 4°C. The supernatant was discarded, and the salt-washed membrane pellet was resuspended by using homogenizer in 25 mM MOPS buffer (pH 7.0) (membrane fractions) and stored at –80°C under nitrogen.

pMMO assay

pMMO activity was measured by propene epoxidation (Burrows *et al.* 1984). The sample solution (3.5 ml) contained membrane fractions and reducing reagents in 25 mM MOPS buffer (pH 7.0). The flask (*c.* 10 ml) was sealed with a Teflon-sealed septum, and incubated for 5 min at reaction temperature. The reaction was initiated by injecting 2.5 ml of propene into the flask with a gas-tight syringe. The produced propene epoxide was measured by gas chromatography using a Sorbitol 25%–Gasport B column (4 m × 3 mm i.d., GL Sciences Inc., Tokyo, Japan) attached to a Hitachi 263-30 gas chromatograph (Hitachi Ltd., Tokyo, Japan) (oven temperature, 100°C; carrier gas, N_2; flow rate, 21.8 ml min^{-1}).

Specific activity was obtained by dividing the activity by the total amount of protein in the sample determined by the Lowry method (Lowry *et al.* 1951).

Preparation of quinols

Reduction of quinones to quinols was performed by a modification of the method described by Shiemke *et al.* (1995). All procedures were performed under anaerobic conditions by adding excess sodium dithionite and purging nitrogen gas.

ESR spectroscopy

ESR specta were recorded on a JEOL RE1X ESR spectrometer (JEOL, Tokyo, Japan). The ESR samples were prepared by sealing 500 μl of membrane fractions under argon atmosphere in quartz ESR tubes. To prepare the samples, the membrane fractions were rapidly frozen in cooled heptane and liquid nitrogen.

Results and discussion

Effect of copper (II) concentration in the growth medium

M. trichosporium OB3b was cultivated in mineral salts media containing copper (II) sulfate. As shown in

Table 1. Effect of copper (II) concentration in the culture medium on growth rate and pMMO activity

Copper (II) concentration (μM)	Biomass (mg dry-cell ml^{-1})	Specific growth rate (min^{-1})	MMO activity (μmol min^{-1} g^{-1} dry-cell)
< 0.21	0.217	1.18×10^{-3}	27.0
1.25	0.235	1.59×10^{-3}	64.9
20	0.122	1.39×10^{-4}	98.6

Table 1, the MMO activity in cells cultivated with 20 μM of copper (II) concentration was higher than the activity with 1.25 μM of copper (II) concentration. However, the growth rate of *M. trichosporium* OB3b and the maximum cell density decreased with increasing copper (II) concentration up to 20 μM. The maximum specific growth rate was 1.59×10^{-3} min^{-1} and the maximum cell density was 0.235 mg dry-cell ml^{-1} at 1.25 μM of copper (II) concentration. Thus, *M. trichosporium* OB3b was cultivated as described in Materials and methods. A final medium containing 10 μM $CuSO_4$ was used throughout this work.

Reduction of pMMO by quinols

For hydroxylation, pMMO requires reducing agents. Quinols can provide reducing equivalents for pMMO from *Methylococcus capsulatus* (Bath), substituting for NADH (Shiemke *et al*. 1995). In this study, it was found that quinols also provided reducing equivalents for pMMO from *M. trichosporium* OB3b. As shown in Table 2, the activity of pMMO in membrane fractions was highest when duroquinol was used, but duroquinol was not as effective as NADH for the activity in whole cells. Although the solubility of the quinols is low in aqueous solution, duroquinol was more effective than NADH in membrane fractions. Other quinols were not as effective as duroquinol, despite their structural similarity. Although trimethylquinol was effective for pMMO from *M. trichosporium* OB3b, it was ineffective for pMMO from *M. capsulatus* (Bath) (Shiemke *et al*. 1995).

Stabilization of pMMO in membrane fractions

pMMO activity in membrane fractions is extremely labile. Consequently, pMMO has not yet been purified. In this study the effects of temperature and additives on the stability of pMMO in membrane fractions from *M. trichosporium* OB3b were measured.

Figure 1 shows the effect of BSA on the stability of pMMO at 4°C. When pMMO was stored at 4°C, more than 50% of the activity was lost within 24 h. However, more than 90% of the activity freshly prepared was retained more than one month when the membrane fraction was rapidly frozen at –196°C under purged nitrogen gas and stored at –80°C (data not shown).

BSA is known as the most effective additive for ammonia monooxygenase from *Nitrosomonas europaea*, to stabilize ammonia-dependent O_2 uptake activity (Juliette *et al*. 1995). In this case a little loss of pMMO activity in membrane fractions was also observed. In the presence of BSA (10 mg ml^{-1}), as shown by closed circles in Figure 1, BSA stabilized pMMO activity in membrane fractions for up to 24 h at 4°C.

Effect of additives on pMMO activity

The effects of metal ions and BSA for pMMO activity are shown in Table 3. pMMO activity was strongly influenced by the addition of metal ions and BSA. The copper (II) ion also inhibited pMMO from the membrane fractions as described in Material and methods. However, when the cells

Table 2. Comparison of pMMO specific activity in membrane fractions with various substituted *p*-benzoquinols

Reductant[1]	Whole cells (nmol min^{-1} mg^{-1} dry-cell)	Membrane fractions (nmol min^{-1} mg^{-1} protein)	Redox potential[2] ($E^{0'}$)(mV)	Hydrophobicity[3]
No addition	5.53[4]	0	–	–
NADH	77.9[5]	1.07	–340	Not determined
Decyl-plastoquinol	–	0.342	30	97.9 ± 0.008
Duroquinol	5.27[4]	4.79	50	96.7 ± 0.005
Coenzyme Q_0	–	0.274		90.4 ± 0.02
Menaquinol	–	0	0	96.8 ± 0.008
2,3-Dimethylquinol	–	0	210	96.3 ± 0.002
Trimethylquinol	–	0.653	115	96.4 ± 0.006

[1]Reductants were present at a concentration of 10 nM for membrane fractions except for decyl-plastoquinol (7.7 mM).
[2]$E^{0'}$ values from R.M.C. Dawson *et al*., 'Data for Biochemical Research', 3rd edn, Clarendon Press, Oxford (1986).
[3]The octanol–water partition coefficients for the quinols were determined at room temperature. Hydrophobicity values from A.K. Shiemke *et al*., *Arch Biochem Biophys* **321** (1995) 421–428.
[4]Activity assayed with 183 mM dimethyl sulfoxide.
[5]Activity assayed with 14 mM sodium formate.

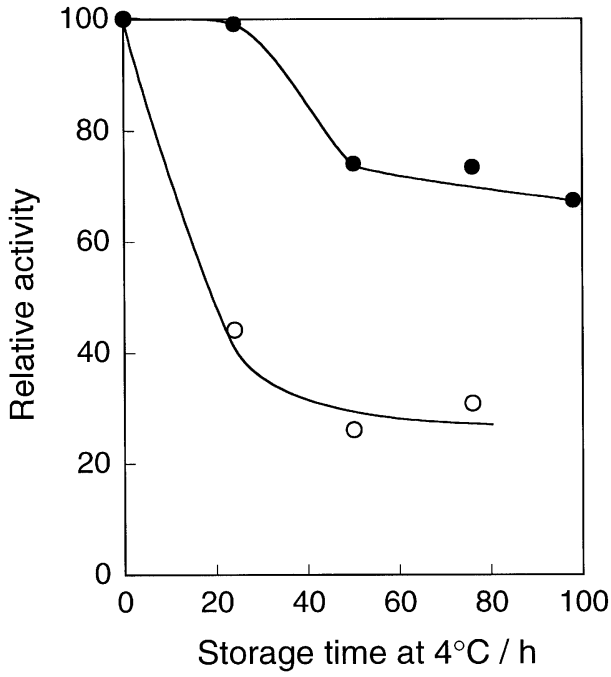

Figure 1. pMMO activity in membrane fractions measured in the presence and absence of BSA. The activity of membrane fractions was assayed at the indicated times as described in Materials and methods in the presence of 10 mg ml^{-1} BSA (●) or absence of BSA (○).

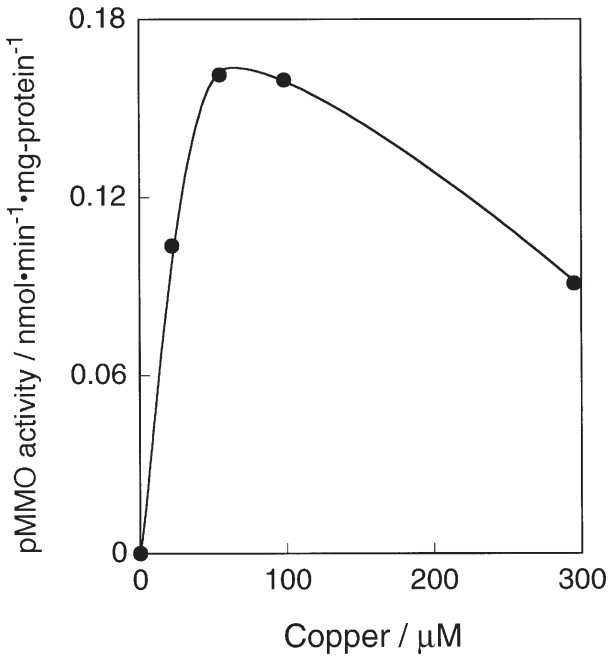

Figure 2. The effect of exogenous copper (II) ion on pMMO activity in membrane fractions.

were broken in the absence of copper (II), the copper (II) ion stimulated pMMO activity as shown in Figure 2. The activity increased with the concentration of copper (II) ion and then decreased through a maxiumum point. Maximum pMMO activity was obtained using 100 μM of copper (II) concentration. These results indicate that copper (II) is needed for the active sites in pMMO from *M. trichosporium* OB3b but that excess copper (II) ions inhibit pMMO activity.

pMMO activity in membrane fractions was inhibited by EDTA, dithiothreitol (threo-1,4-dimercapto-2, 3-butanediol), methanol, thiourea, azide, myxothiazol and acetylene (Table 4). Although the pMMO from *M. capsulatus* (Bath) ws stimulated by myxothiazol, pMMO activity from *M. trichosporium* OB3b was inhibited by myxothiazol. Treatment of the membrane fraction with acetylene, dioxygen and duroquinol resulted in irreversible inhibition of pMMO.

Table 3. Effect of metal ions and proteins on propene epoxidation with the membrane fractions from *M. trichosporium* OB3b

Additive	Concentration	Specific activity (nmol min^{-1} mg^{-1} protein)	Relative activity (% of control)
No addition		4.07	100
$Cu(II)Cl_2$	300 μM	1.24	30.4
$Fe(II)SO_4$	300 μM	4.62	114
$Fe(III)Cl_3$	300 μM	4.26	105
BSA[1]	10 mg ml^{-1}	6.06	149
Ovalbumin	10 mg ml^{-1}	3.38	83.2
PMSF[2]	300 mM	3.95	97.2
Acetone[3]	10.3 mM	4.00	98.1

[1] Bovine serum albumin.
[2] Phenylmethylsulfonyl fluoride.
[3] Solvent for PMSF stock solution.

Table 4. Effect of inhibitors on propene epoxidation by membrane fractions from *M. trichosporium* OB3b

Inhibitor	Concentration (mM)	Specific activity (nmol min^{-1} mg^{-1} protein)	Relative activity (%)
No addition	–	4.97	100
EDTA	0.5	4.12	82.9
	2.0	3.53	71.0
	8.0	3.61	72.6
Dithiothreitol	0.1	3.72	74.8
	1.0	0	0
Thioruea	0.5	1.79	36.0
	1.0	1.11	22.3
Sodium azide	1.0	3.05	61.4
	2.0	2.34	47.1
Methanol	1.0	4.41	88.7
	9.0	2.34	47.1
Myxothiazol*	0.05	4.65	93.6
	0.1	4.72	95.0
	0.2	3.91	78.7
Acetylene	17% (vol/vol)	0	0

*Solvent (acetone) was removed by evaporation under vacuum.

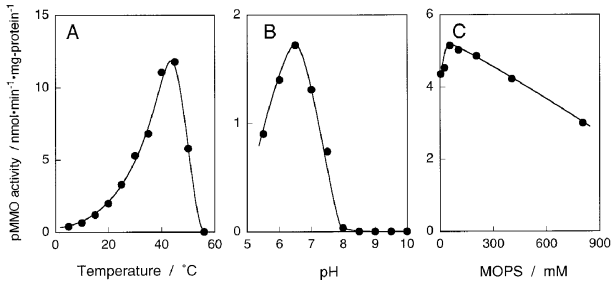

Figure 3. Factors affecting pMMO activity in membrane fractions. A, the effect of reaction temperature; B, the effect of pH, C, the effect of buffer concentration.

Methanol is the product of methane hydroxylation by pMMO. It was known that an electron donor such as exogenously supplied methanol is required for the oxgenation of n-alkanes and n-alkenes with methanotrophs (Hou *et al.* 1980). The results presented here, however, indicate that the propene epoxidation activity is inhibited by methanol, i.e. pMMO was inhibited by methanol.

Optimization of pMMO activity assay

To optimize propene epoxidation with membrane fractions from *M. trichosporium* OB3b, the effects of reaction temperature, pH and concentration of MOPS buffer (pH 7.0) were examined.

The effect of reaction temperature on propene epoxidation with membrane fractions indicated the optimum temperature to be 45°C (Figure 3A). Although the freshly prepared pMMO in membrane fractions lost more than 50% of their activity within 24 h when stored at 4°C, the optimum temperature of pMMO activity may have been much higher. Increasing the temperature results in a decrease in pMMO activity, possibly due to instability of the enzyme pMMO.

Figure 3B shows the effect of pH on propene epoxidation with membrane fractions. Good's buffer (MES, MOPS, Tris and glycine, concentration of 25 mM) was used for pH values from 5.5–10.0. The optimum pH was 6.5, which is lower than the pH 7.5 optimum in pMMO from *M. capsulatus* (Bath) (Zahn *et al.* 1996).

The effect of concentration of MOPS buffer (pH 7.0) on propene epoxidation with membrane fractions is shown in Figure 3C, indicating that propene epoxidation was stimulated at low MOPS concentration, but that the propene epoxidation activity was obtained using 55 mM MOPS buffer pH 7.0).

In conclusion, the optimum conditions for pMMO activity in membrane fractions were reaction temperature 45°C, pH 6.5 and 55 mM MOPS buffer and the rate of propene oxide formation was 13.6 nmol min^{-1} mg^{-1} protein in these optimum conditions.

ESR spectra of membrane fractions from M. trichosporium *OB3b*

Figure 4 shows the ESR spectra of membrane fractions from *M trichosporium* OB3b. This ESR spectrum indicated a major type II copper (II) signal ($g_{//} = 2.24$, $A_{//} = 18.4$ mT, $g_{\perp} = 2.06$) and a minor $g = 4.28, 5.98$.

Nguyen and co-workers reported that anaerobic titration of highly oxidized membranes from *M. capsulatus* (Bath) by dithionite leads to partially reduced copper cluster species and a substantial decrease in the intensity of the copper (II) ESR signal (Nguyen *et al.* 1994). As shown in Figure 5, anaerobic and successive addition of excess amounts of duroquinol resulted in a decrease in the intensity of the copper (II) ESR signal and the appearance of the multiple hyperfine structure at g = 2.05 (Figure 5B). Upon exposure of this reduced sample to dioxygen, the copper (II) ESR signal increased (Figure 5C). The ESR spectra indicated that the copper cluster in membrane fractions is

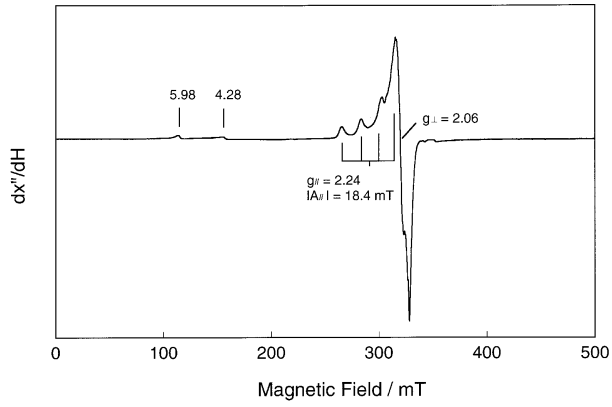

Figure 4. X-band ESR spectra of membrane fractions from *M. trichosporium* OB3b. The spectra were recorded at 7.10 K with 0.20 mW of microwave power, modulation amplitude of 0.5 mT, modulation frequency of 100 kHz, time constant of 0.03 s, and gain of 125. The microwave frequency was 8.988 GHz.

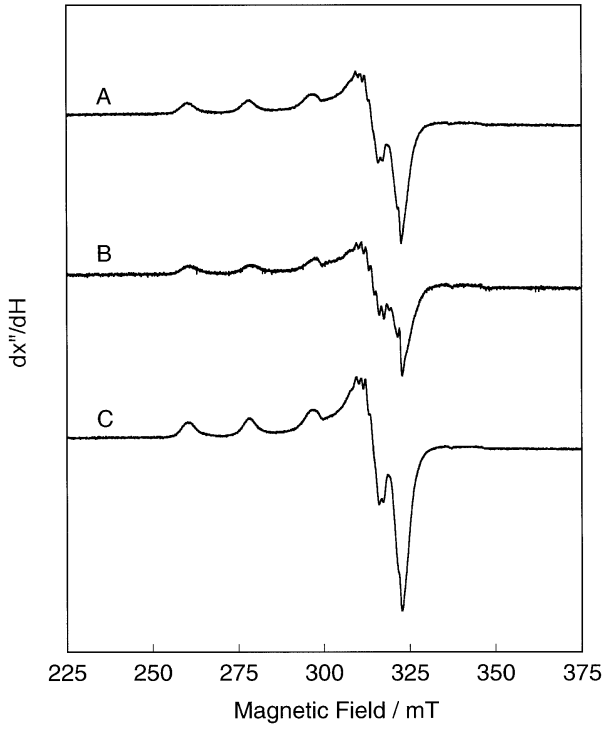

Figure 5. X-band ESR spectra of membrane fractions from *M. trichosporium* OB3b during the course of anaerobic redox titration with duroquinol and after exposure to dioxygen. A, before addition of duroquinol under anaerobic conditions; B, after addition of duroquinol; C, after exposing the sample (B) to dioxygen for 10 min at room temperature. ESR spectra were recorded at 79.0 K with 1.00 mW of microwave power, modulation amplitude of 0.5 mT, modulation frequency of 100 kHz, time constant of 0.03 s, and gain of 125. The microwave frequency was 9.003 GHz.

reduced and oxidized by duroquinol as an optimum reductant of pMMO, and dioxygen as a substrate of pMMO, respectively. The result suggests that the copper cluster is contained in the active site of pMMO.

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