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Effect of bovine serum albumin on particulate methane monooxygenase from *Methylosinus trichosporium* OB3b

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Abstract

The effect of some additives on the membrane bound particulate methane monooxygenase (pMMO) from *Methylosinus trichosporium* OB3b was studied. The pMMO activity was stimulated by exogenously supplied bovine serum albumin (BSA), but was inhibited by ovalbumin and excess copper (II) ion. When the membrane was treated with BSA, the amount of copper (II) ion in the membrane decreased, but the specific activity increased about 1.8 times compared with the as-isolated membrane. BSA may remove excess copper (II) ions in the membrane, inhibiting the pMMO activity. When the excess copper is removed by BSA, pMMO activity is stimulated.

Keywords: Particulate methane monooxygenase; *Methylosinus trichosporium* OB3b; Copper; Bovine serum albumin

1. Introduction

Methane monooxygenase (MMO) in the methanotrophs catalyzes the hydroxylation of methane to methanol. The type of MMO, soluble or membrane-bound, strongly depends on the copper (II) concentration during the growth of *Methylosinus trichosporium* OB3b [1]. At low copper-to-biomass ratios, the enzyme activity is mainly in the soluble fraction and is referred to as the soluble MMO (sMMO). At higher copper-to-biomass ratios, methane hydroxylation is catalyzed in the membrane fraction by the membrane-bound or particulate MMO (pMMO). Though sMMO has been purified and studied extensively both on the biochemical and the genetic levels, there are few data available about pMMO. As we reported previously, some additives stimulated the pMMO activity. Among the additives BSA showed strong effect to increase the activity. In this study, the effect of BSA on the pMMO activity in the membrane from *M. trichosporium* OB3b was studied, and the role of BSA in the activity was discussed.

Abbreviations: MMO, methane monooxygenase; pMMO, particulate methane monooxygenase; sMMO, soluble methane monooxygenase; AMO, ammonia monooxygenase; BSA, bovine serum albumin; MOPS, 3-morpholinopropanesulfonic acid; ESR, electron spin resonance; EDTA, ethylenediaminetetraacetic acid

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2. Materials and methods

2.1. Materials

Methane and propene were purchased from Fujiibussan (Tokyo, Japan). Bovine serum albumin (BSA), ovalbumin, duroquinone (tetramethyl-*p*-benzoquinone) were obtained from Sigma–Aldrich Japan (Tokyo, Japan). 3-Morpholinopropanesulfonic acid (MOPS) was obtained from Wako (Tokyo, Japan). Copper standard solution for atomic absorption spectrometry, ethylenediaminetetraacetic acid and the other chemicals were purchased from Kanto Chemical (Tokyo, Japan). The other chemicals were of the highest grade available and were used without further purification.

2.2. Culture of *M. trichosporium* OB3b

M. trichosporium OB3b was kindly provided by Professor J.D. Lipscomb of University of Minnesota. *M. trichosporium* OB3b cells were prepared as previously described [2]. pMMO activity was measured by propene epoxidation [3]. Specific activity was obtained by dividing the activity by the total amount of protein in the sample determined by Lowry method.

2.3. Isolation of membrane 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 anaerobically. Just prior to break the cells the suspension was supplemented with 300 μM $CuSO_4$ and with DNase I (10 μg ml-suspension^{-1}). During breaking 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 membrane was 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), and the membrane fractions were stored at −80°C under nitrogen.

2.4. Treatment of membrane with BSA and EDTA

The membrane fractions were incubated with BSA (1.19 mg-BSA mg-protein^{-1}) or EDTA (157 μmol mg-protein^{-1}) for 30 min at 4°C. To remove excess additives and chelated metal complex, the solution was centrifuged at $143{,}000 \times g$ for 90 min at 4°C. The supernatant was discarded, and the membrane was resuspended by using homogenizer in 25 mM MOPS buffer (pH 7.0) and centrifuged at $143{,}000 \times g$ for 90 min at 4°C. The supernatant was discarded, and membrane was resuspended by using homogenizer in 25 mM MOPS buffer (pH 7.0) and stored at −80°C under nitrogen.

2.5. Copper ion analysis

The membrane was heated at 94°C with distilled water containing 1.0 N NaOH for 5 min prior to copper ion analysis. Copper was analyzed by atomic absorption spectroscopy on a Shimadzu AA-625-11 (Kyoto, Japan). The metal concentration of the samples was determined on the reference of standard solution.

2.6. ESR spectroscopy

ESR spectra were recorded on a JEOL RE1X ESR spectrometer (Tokyo, Japan). The ESR samples were prepared by 500 μl of samples in quartz ESR tubes. Then, the samples were rapidly frozen in cooled *n*-heptane and liquid nitrogen.

3. Results and discussion

3.1. The role of bovine serum albumin on particulate methane monooxygenase activity in the membrane

As we reported previously, some additives increased the pMMO activity in the membrane [2]. Among the additives BSA showed a remarkable effect. Fig. 1 shows time dependence of propylene oxide formation, showing BSA is effective. On the other hand, another protein, ovalbumin, inhibits the activity a little, indicating this effect may be specific for BSA. As BSA has one specific copper (II) binding site [4], and BSA binding copper (II) is one of the transportation forms of copper in the blood. The effect of BSA on pMMO activity in the membrane may be due to the copper-binding effect of BSA. BSA may extract an excess copper (II) in the membrane.

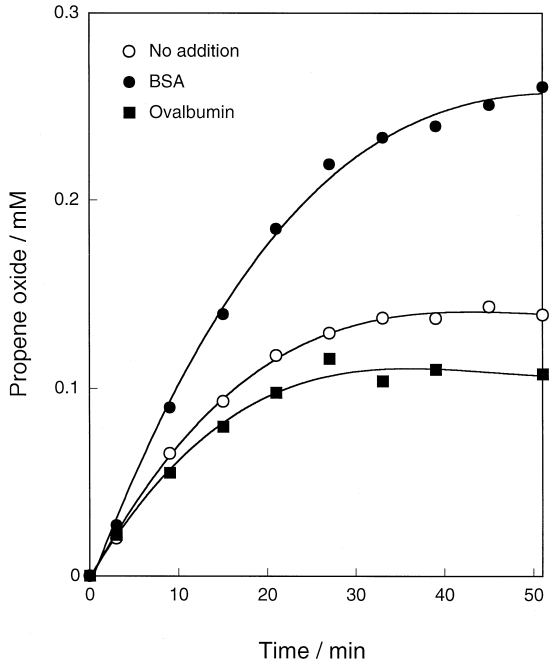


Fig. 1. The effect of exogenous bovine serum albumin and ovalbumin on pMMO activity in the membrane. The reaction mixture contains membrane (1.6 mg-protein ml^{-1}), duroquinol (25.3 μmol), propene (112 μmol) and oxygen (103 μmol) in 25 mM MOPS buffer (pH 7.0). The reaction was carried out at 30°C.

Table 1
The amount of copper in the BSA-treated membranes and supernatant

Sample	Copper (μmol)	Yield	Specific activity (nmol min^{-1} mg^{-1})
As-isolated membranes	11.8	100%	1.16
BSA-treated membranes	8.9	75%	2.04
BSA-treated supernatant	2.5	21%	0

The membrane (7.02×10^{-2} mg-protein) from *M. trichosporium* OB3b contains unusual large amount of copper, 11.8 μmol-copper. When the membrane was treated by BSA, the specific activity increased about 1.8 times, and the amount of copper ion in the BSA-treated membrane decreased by about 20% of as-isolated membrane as shown in Table 1. The result shows indicated that the specific activity of the BSA-treated membrane increases instead of the decrease of copper amount in the membrane, and suggests that excess copper ions in the membrane are extracted by BSA, and the pMMO activity is stimulated.

The effect of the other copper (II) chelator such as EDTA on the pMMO activity in the membrane was investigated. Table 2 shows the

Table 2
Effect of BSA and EDTA on the pMMO activity and copper content

Sample	Specific activity (nmol min^{-1} mg-protein^{-1})	Relative activity	Copper (μmol mg-protein^{-1})
Pre EDTA-treated membranes	1.74	100%	94.4
EDTA-treated membranes	1.43	82.2%	54.3
Pre BSA-treated membranes	1.45	100%	125
BSA-treated membranes	2.55	176%	1.22

effect of EDTA and BSA on copper content and pMMO activity in the membrane. When the membrane was treated with EDTA, the specific activity decreased a little and copper content (copper/protein ratio) decreased. The result suggests that EDTA may extract a required metal cofactor such as the active site of pMMO. When the membrane was treated by BSA, however, the specific activity increased and copper per mg-protein did not change as shown in Table 2. The result suggests that the BSA-treated supernatant contains BSA–copper complex and some components of membrane such as proteins, peptides, coenzymes or lipids.

Fig. 2 shows the ESR spectrum and ultraviolet absorption spectrum of the BSA-treated supernatant. The ESR spectrum of the BSA-treated supernatant had a square-planar type ESR spectral pattern (Fig. 2a) with ESR parameters: $g_{//} = 2.15$ and $A_{//} = 21.3$ mT. This ESR spectrum was similar to the ESR spectrum of BSA–copper (II) complex with ESR parameter: $g_{//} = 2.18$ and $A_{//} = 21.8$ mT [5]. Fig. 2b shows the ultraviolet absorption spectra of the BSA-treated supernatant, BSA and BSA–copper complex. BSA–copper complex was prepared by mixing BSA (15.2 μM) and Cu (15.5 μM) in 25 mM MOPS (pH 7.0) at 30°C for 30 min. The absorption spectrum of the BSA-treated supernatant has a broad band with a distinct shoulder at 259 nm (Fig. 2b, solid line). On the other hand, the absorption spectra of BSA and BSA–copper complex had the maximum wavelength at 278 nm and 277 nm, respectively. The result suggests that the complex in the supernatant contains BSA, copper and also some other components such as proteins, peptides, coenzyme or lipids.

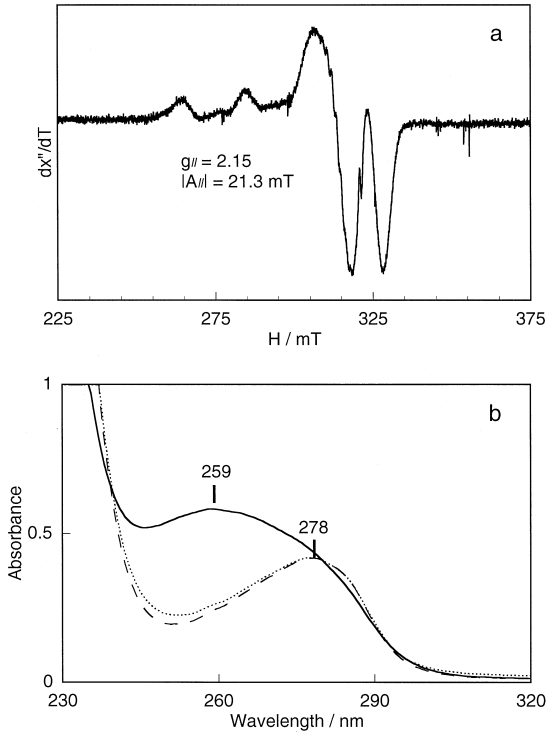


Fig. 2. ESR spectrum and ultraviolet absorption spectrum of the BSA-treated supernatant. (a) X-band ESR of the BSA-treated supernatant. ESR spectrum was recorded at 77.3 K with 1.00 mW of microwave power, modulation amplitude of 0.5 mT, modulation frequency of 100 kHz, time constant of 0.03 s, and gain of 320. The microwave frequency was 9.003 GHz. (b) Ultraviolet absorption spectra of the BSA-treated supernatant (————), BSA (– – –) and BSA–copper (II) complex (· · ·).

3.2. Inactivation of particulate methane monooxygenase by copper and its protection by bovine serum albumin

The effect of copper (II) ion against membrane from *M. trichosporium* OB3b was studied. Addition of copper (II) ion to the membrane leads to inactivation of the enzyme (Fig. 3, closed circle). However, copper ion is needed for the activity of pMMO from *M. trichosporium* OB3b [6–8]. Copper in the membrane may play as active site of pMMO or electron transfer, but excess copper leads to inactivation of the enzyme. This abnormal phenomenon is also observed in other methanotrophs, but the reason for the inhibition of the pMMO activity by excess of copper is not clarified yet [9,10]. Excess copper (II) ion may displace other metals from the active site of a reductase, electron transfer, or monooxygenase in pMMO.

Fig. 3 shows the effect of BSA on pMMO activity in the membrane in the presence of copper. The pMMO activity decreases with the increase of copper concentration without BSA,

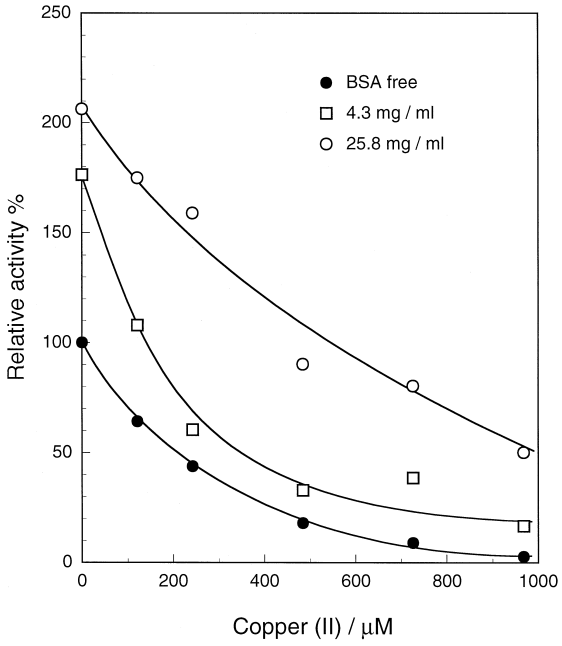


Fig. 3. The effect of exogenous copper (II) ion on pMMO activity in the presence and absence of bovine serum albumin. The reaction mixture contains membrane (2.6 mg-protein ml^{-1}), duroquinol (25.3 μmol), propene (112 μmol) and oxygen (103 μmol) in 25 mM MOPS buffer (pH 7.0). The reaction was carried out at 30°C.

but the activity was observed for up to 968 μM of copper concentration with BSA (4.3 mg ml^{-1}). These results suggested that the effect of BSA on pMMO activity in the membrane may be due to copper-binding effect of BSA, i.e., excess copper (II) ions in the membrane, inhibiting pMMO activity, were extracted BSA, and the pMMO activity in the membrane was stimulated.

3.3. Two types of copper in the membrane from M. trichosporium OB3b

Fig. 4 shows the ESR spectra of as-isolated, BSA-treated and EDTA-treated membrane at 78 K. As shown in Fig. 4a, the ESR spectrum of the membrane shows a typical type II copper (II) signal ($g_{//} = 2.24$, $A_{//} = 18.4$ mT, $g_{\perp} = 2.06$). When the membrane was treated by BSA, the copper ESR signal did not change (Fig. 4b), indicating that the copper cluster in BSA-treated membrane is preserved in spite of a decrease in copper. However, the treatment of the membrane by EDTA resulted in a decrease in the intensity of the copper ESR signal and then the appearance of multiple hyperfine structure ($|A| = 1.45$ mT) at $g = 2.06$ (Fig. 4c). This distinction of these spectra indicated that copper in the membrane has two types, one type of the copper functions as active site of pMMO or regulatory of the activity, and the other type of copper inhibits the pMMO as shown in Scheme 1. When the membrane is treated by EDTA, both types of copper in the membrane are extracted, and pMMO activity and copper contents in the membrane decrease. When the membrane is treated by BSA, excess copper ions inhibiting pMMO activity are extracted and some compo-

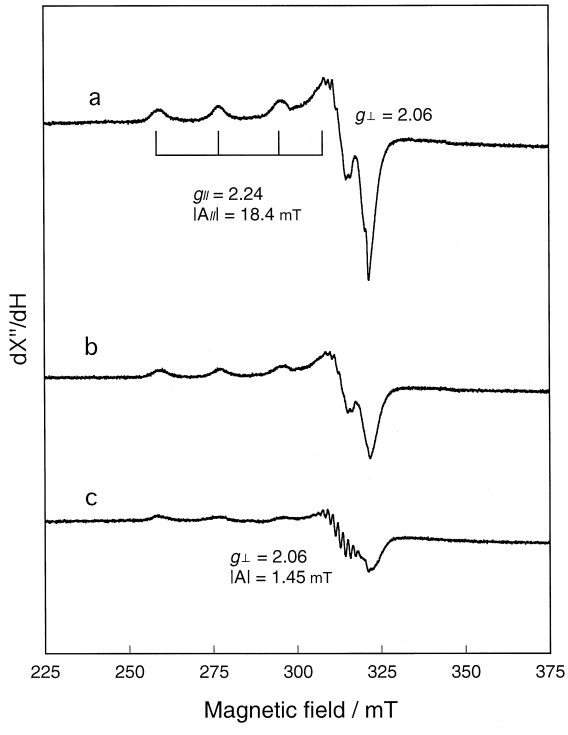
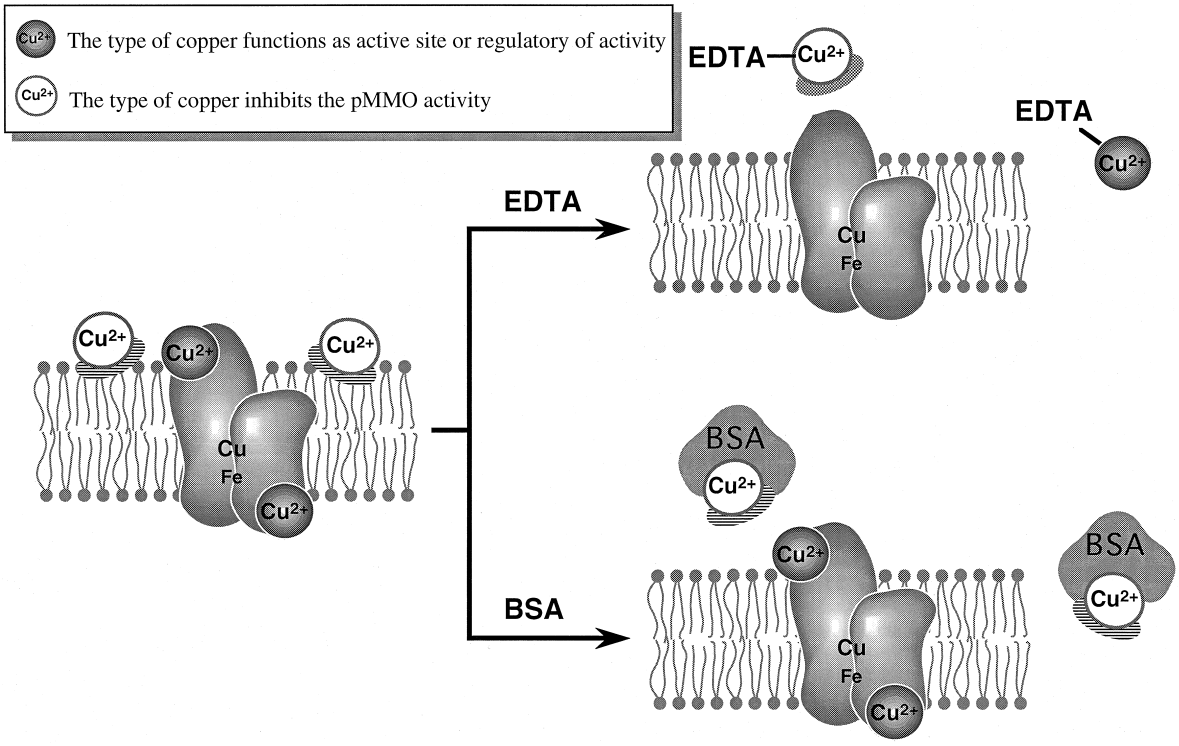


Fig. 4. X-band ESR spectra of the BSA-treated and EDTA-treated membrane. (a) As-isolated membrane from *M. trichosporium* OB3b. (b) BSA-treated membrane. (c) EDTA-treated membrane. ESR spectrum was recorded at 79.0 K with 1.00 mW of microwave power, modulation amplitude of 0.5 mT, modulation frequency of 100 kHz and time constant of 0.03 s, and gain of 160. The microwave frequency was 9.003 GHz.



Scheme 1. Hypothetical scheme for the interaction of copper clusters in pMMO-containing membranes with BSA.

nents in the membrane, and pMMO activity in the membrane increases. Thus, BSA only extracts an excess copper ions inhibiting pMMO activity but does not extract a required copper for the activity of pMMO, and EDTA extracts an extract excess copper ions and copper cluster such as active site of pMMO or regulatory of the activity.

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