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Role of iron in particulate methane monooxygenase from *Methylosinus trichosporium* OB3b

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The effect of iron ions on particulate methane monooxygenase was studied by using the EDTA-treated membranes from *Methylosinus trichosporium* OB3b. When the membrane was treated with EDTA the activity remained 82% of the as-isolated membranes, and the activity of the EDTA-treated membranes was strongly influenced by the addition of metal ions. Among the metal ions, ferric, ferrous and cupric ions stimulated the activity, indicating those ions were needed for the activity. When propargylamine was added, pMMO activity decreased and also the iron ESR signal decreased. As the ESR signal involves the ferrous nitrosyl complex in EDTA-treated membranes, the active site of pMMO may contain a mononuclear non-heme iron.

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

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

Introduction

Methane monooxygenase (MMO) in methanotrophs catalyzes the hydroxylation of methane to methanol. The formation of a soluble or membrane-bound MMO strongly depends on the copper concentration during the growth of *Methylosinus trichosporium* OB3b (Scott *et al.* 1981). At low copper-to-biomass ratios, the enzyme activity is in the soluble fraction and the enzyme is referred to as 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 in the biochemical and the genetic levels, there are few data available about pMMO, because of the instability.

Recently, pMMO from *Methylococcus capsulatus* (Bath) and *M. trichosporium* OB3b has been purified and it is clarified that the enzyme contains both copper and iron (Zahn & DiSpirito 1996, Nguyen *et al.* 1998, Takeguchi *et al.* 1998a). By the copper concentration dependence of pMMO activity and the ESR spectrum (Nguyen *et al.* 1994), it is proposed that the active site of pMMO from *M. capsulatus* (Bath) is a copper cluster. Though Nguyen and co-workers reported the presence of two types of copper ions (E-cluster and C-cluster) in pMMO from *M. capsulatus* (Bath) (Nguyen *et al.* 1996), the role of iron in pMMO is not clarified yet.

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As we reported previously the pMMO activity in the membrane was inhibited by ethylenediaminetetraacetic acid (EDTA), but the pMMO activity remained 82% of the as-isolated membranes (Takeguchi *et al.* 1998c), suggesting that the EDTA-treated membrane contains the active site of pMMO from *M. trichosporium* OB3b. The ESR spectrum of pMMO from *M. trichosporium* OB3b had a type 2 copper signal and a weak high-spin iron signal (Takeguchi *et al.* 1998a). In this paper, we describe the effect of iron on the membranes from *M. trichosporium* OB3b treated by EDTA, and the role of iron is discussed.

Materials and methods

Materials

Methane and propene were purchased from Fujiibussan Co. (Tokyo, Japan). Duroquinone (tetramethyl-*p*-benzoquinone), phenazine ethosulfate, phenosafranin, safranin O, neutral red, benzyl viologen and methyl viologen were obtained from Sigma-Aldrich Japan K. K. (Tokyo, Japan). Anthraquinone 2-sulfonate and 3-morpholinopropanesulfonic acid (MOPS) were obtained from Wako Pure Chemical Industries (Tokyo, Japan). Methylene blue, indigodisulfonate, 2-hydroxy-1, 4-naphthoquinone, quinhydrone, disodium dihydrogen ethylenediaminetetraacetate dihydrate, ferric chloride hexahydrate, ferrous chloride tetrahydrate and cupric chloride dihydrate were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Resorufin was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Propargylamine hydrochloride was purchased from Tokyo Chemical Industry Co., LTD. (Tokyo, Japan). The other chemicals were of the highest grade available and were used without further purification.

Isolation of membranes from M. trichosporium OB3b

M.trichosporium OB3b was kindly provided by Professor J. D. Lipscomb of University of Minnesota. *M. trichosporium* OB3b cells and membranes of the cell were prepared as previously described (Takeguchi *et al.* 1998b). pMMO activity was measured by propene epoxidation (Burrows *et al.* 1984). Specific activity was obtained by dividing the activity by the total amount of protein in the sample determined by Lowry method (Lowry *et al.* 1951).

Treatment of membranes with EDTA

The membranes were incubated with 14.3 mM EDTA for 30 min at 4°C. To remove excess EDTA and EDTA chelated metal complex, the solution was centrifuged at 143,000 × 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) and centrifuged at 143,000 × 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) and stored at –80 °C under nitrogen.

Metal ion analysis

The membranes were heated at 94°C with distilled water containing 1.0 N NaOH for 5 min prior to metal ion analysis. Copper ion analysis was performed by atomic absorption spectroscopy on a AA-625–11(Shimadzu, Tokyo, Japan). The copper concentration of the samples was determined on the reference solution purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). A solution of 1.0 N NaOH in distilled water was used as a copper-free control.

ESR spectroscopy

X-band ESR spectra were recorded on a JEOL RE1X ESR spectrometer (JEOL, Tokyo, Japan) at liquid nitrogen and liquid helium temperature. The ESR samples were prepared by sealing 500 μl of membranes under argon atmosphere in quartz ESR tubes. To prepare the samples, the membranes were rapidly frozen in cooled *n*- heptane and liquid nitrogen.

Redox titration

The redox titration was carried out at 30 °C in a device adapted from the design of Dutton (Dutton 1978). Potential measurements were made using a pH meter F–14 (Horiba, Kyoto, Japan). The cell, equipped with a platinum and a calomel standard electrode, was calibrated with a saturated solution of quinhydrone in 25 mM MOPS buffer (pH 7.0). During the titrations, the system was kept anaerobic by a constant purge of argon gas. The following mediators (16 μM each) were present in the titration: phenazine ethosulfate (+55 mV), methylene blue (+11 mV), resorufin (–51 mV), indigodisulfonate (–125 mV), 2-hydroxy–1, 4-naphthaquinone (–145 mV), anthraquinone 2-sulfonate (–225 mV), phenosafranin (–252 mV), safranin O (–280 mV), neutral red (–340 mV), benzyl viologen (–350 mV), and methyl viologen (–440 mV). All potentials are referenced to a normal hydrogen electrode (NHE). For an exploratory titration run, mediators covering a wide range of potentials (+150 to –420 mV) are used. Sodium dithionite solution (100 mM) in 25 mM MOPS buffer (pH 7.0) was used as a reductant. After the addition of reductant, the titration solution was allowed to stabilize for about 20 min. The sample was withdrawn with a gas-tight syringe and transferred into ESR tube which was attached to the redox vessel. The sample in an ESR tube was quickly frozen in cooled *n*- heptane and liquid nitrogen before the tube was detached from the vessel for ESR measurements. The potentials are reported with respect to a NHE.

Results and discussion

Treatment of membranes with EDTA

As we reported previously the pMMO activity in membranes was partially inhibited by EDTA, for

Table 1. Metals content of membranes fro, M. trichosporium OB3b

Sample	Specific activity /nmol • min^{-1} • mg-protein^{-1}	Copper /mmol • mg-protein^{-1}	Iron /mmol • mg-protein^{-1}	Iron/Copper
As-isolated membranes	1.74	94.4	105.6	1.12
EDTA-treated membranes	1.43	54.3	156.5	2.88

copper (II) ions in membranes were extracted by EDTA (Takeguchi *et al.* 1998c). Although the copper contents in the EDTA-treated membranes decreased by 58% of the as-isolated membranes, the pMMO activity remained 82% of the as-isolated (Table 1). The result suggests that the EDTA-treated membranes contain the active site of pMMO.

On the other hand, the iron content of EDTA-treated membranes increased to 148% of as-isolated (Table 1). This result shows that the treatment of the membranes by EDTA extracts not only metal ions but also membrane-binding proteins. Thus, the iron / copper ratio of EDTA-treated membranes decreased to 2.88 from 1.12 in as-isolated, indicating that only copper ions are extracted from the membranes and iron ions remain.

Since the EDTA-treated membranes contained both copper and iron, the ESR spectrum of the EDTA-treated membranes was measured. Figure 1 shows the ESR spectra of the as-isolated and EDTA-treated membranes from *M. trichosporium* OB3b. The ESR spectrum (Fig. 1, trace A) containing a weak high-spin iron signal (g = 6.01) and the type II copper signal ($g_{||}$ = 2.24, $|A_{||}|$ = 18.4 mT, $g_{\perp}$ = 2.06) was observed in the as-isolated membranes. The treatment of the membranes by EDTA resulted in a decrease in the intensity of the copper ESR signal (Fig. 1, trace B) and then the multiple hyperfine spectrum ($|A|$ = 1.45 mT) at g = 2.06 appeared (Fig. 1, trace C). The high-spin iron signal (g = 6.01), however, did not change, and the intensity of the iron signal with g = 4.32 increased (Fig. 1, trace B). The ESR spectrum of the EDTA-treated membranes shows that both the multiple hyperfine signal at g = 2.06 and the high-spin iron signal are contained in the active site of pMMO from *M. trichosporium* OB3b, so that the activity of the EDTA-treated membrane remained 82% of the as-isolated membranes.

Effect of iron ions on pMMO activity in EDTA-treated membranes

The effect of metal ions on pMMO activity against EDTA-treated membranes was studied. Addition of cupric ion to the EDTA-treated membranes treated with EDTA leads to reactivation of the enzyme (Fig. 2, *inset*). The pMMO activity increased about two times and then decreased with increasing copper concentration. These results indicate that copper ions are needed for the activity of pMMO from *M. trichosporium* OB3b and excess copper ions inhibit pMMO activity.

Addition of ferric and ferrous ions to the EDTA-treated membranes resulted in an increase in pMMO activity (Fig. 2). Maximum pMMO activity was obtained using 120 μM of ferric iron and 60 μM of ferrous iron concentration. However, the pMMO activity decreased with increasing ferrous iron concentration. This phenomenon is also observed in other methanotrophs (Zahn & DiSpirito 1996).

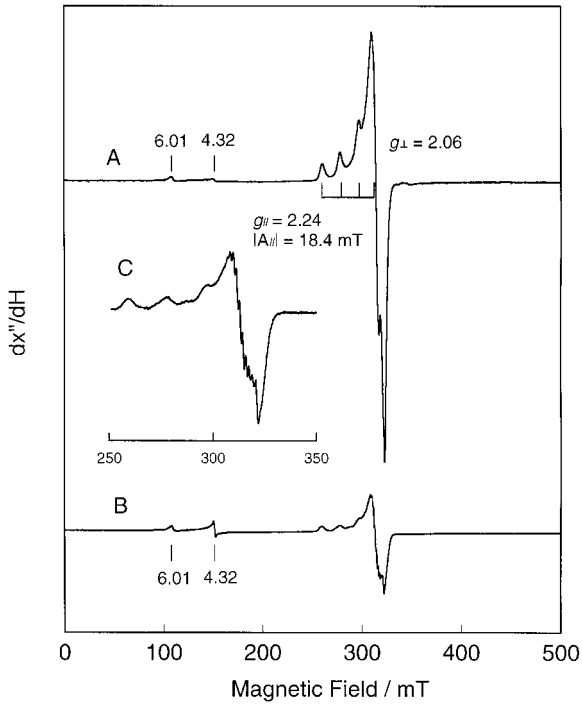

Figure 1. X-band ESR spectra of the as-isolated and EDTA-treated membranes. (Trace A) The membranes obtained from *M. trichosporium* OB3b. (Trace B, C) The membranes treated by EDTA. The spectrum was recorded at 6.7 K with 0.2 mW of microwave power except for trace C (20 μW), modulation amplitude of 1.0 mT, modulation frequency of 100 kHz, time constant of 0.03 s, and gain of 12.5 except for trace C (50).

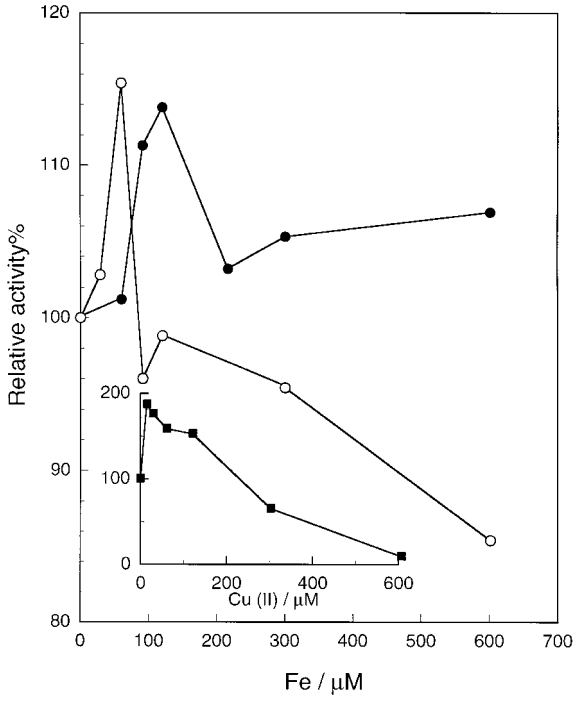

Figure 2. The effect of metal ions on pMMO activity against the EDTA-treated membranes. The effect of exogenous ferrous (open circle) and ferric (closed circle) ions are shown for the membranes that were treated by 14.3 mM EDTA. The inset shows the effect of exogenous cupric ion on pMMO activity. The reaction mixture contains EDTA-treated membranes (1.95 mg-protein ml^{-1}), propene (112 μmol) and oxygen (103 μmol) in 25 mM MOPS buffer (pH7.0). The reaction was carried out at 30°C.

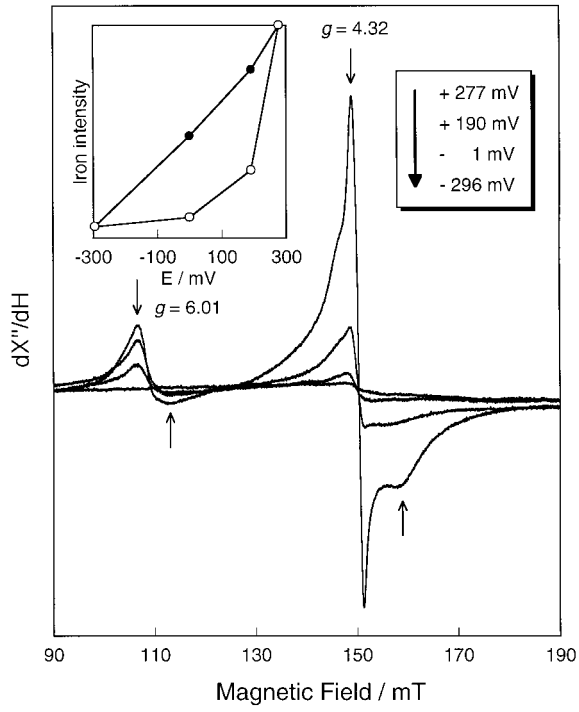

Figure 3. X-band ESR spectra of the EDTA-treated membranes during reductive titration. The potentials at which the samples were poised are indicated. The spectra were recorded at 9.3 K with 2.0 mW of microwave power, modulation amplitude of 1.0 mT, modulation frequency of 100 kHz, time constant of 0.03 s, and gain of 79. The microwave frequency was 9.011 GHz. The inset shows an amplitude of iron ESR signal with g = 6.01 (closed circle) and g = 4.32 (open circle).

These results indicate that copper and iron are needed for the activity of pMMO from *M. trichosporium* OB3b.

Redox behavior of iron in EDTA-treated membranes

The EDTA-treated membranes containing active site of pMMO was used for redox titration of the iron sites. The result is shown in Fig. 3. During the titration, the intensity of the iron signals (g = 6.01 and g = 4.32) decreased with decreasing potential. This phenomenon is also observed in the type 2 copper signal in EDTA-treated membranes (Takeguchi *et al.* 1998d). Redox potential of duroquinol is + 50 mV *vs.* NHE (Shiemke *et al.* 1995). Especially, the intensity of the iron signals decreased during the potential. The result suggested that the iron in EDTA-treated membranes play a role of an electron transfer or an active site of pMMO.

Effect of propargylamine on pMMO activity in EDTA-treated membranes

Acetylene is an inhibitor of sMMO and pMMO (Prior & Dalton 1985). Inactivation of the enzymes is irreversible and is due to binding of the acetylene to the active site of the enzymes, indicating that acetylene acts as a suicide substrate. Since acetylene is a gaseous compound under atmospheric pressure at room temperature, the measurement of acetylene concentration effect is very difficult. Like acetylene, propargylamine also inhibits pMMO specifically, and propargylamine was used in this study. Table 2 shows the effect of propargylamine on pMMO activity in the EDTA-treated membranes. The pMMO activity was inhibited by propargylamine with an increasing concentration, suggesting that propargylamine inhibits pMMO such as acetylene.

Figure 4 shows the effect of propargylamine on ESR spectra of the EDTA-treated membranes from *M. trichosporium* OB3b at 11 K. When duroquinol

Table 2. Effect of propargylamine on pMMO activity in EDTA-treated membranes from *M. trichosporium* OB3b

Propargylamine/mM	Specific activity /nmol • min^{-1} • mg-protein^{-1}	Relative activity
No addition	1.90	100%
16.3	0.93	49%
81.6	0.28	15%

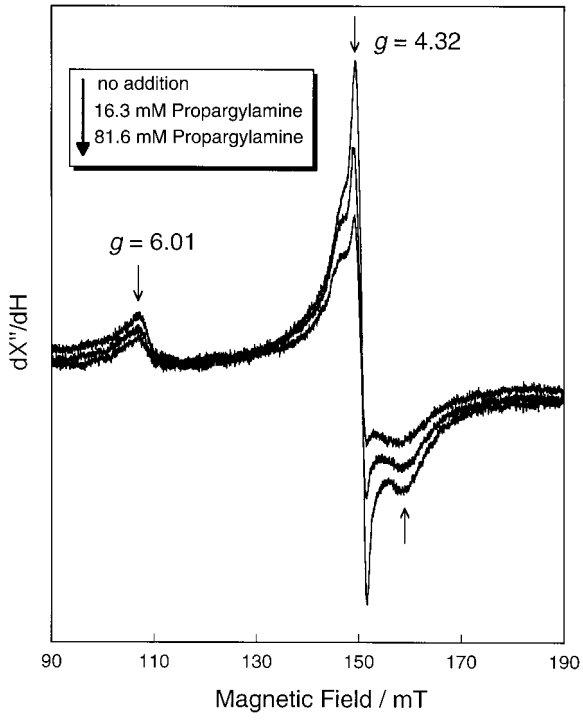

Figure 4 X-band ESR spectra of the EDTA-treated membranes treated by propargylamine. The spectra were recorded at 11 K with 2.0 mW of microwave power, modulation amplitude of 1.0 mT, modulation frequency of 100 kHz, time constant of 0.03 s, and gain of 250.

and propargylamine were introduced to the membranes in aerobic conditions, the iron signal (g = 4.32, 6.01) decreased, but the copper ESR signal containing multiple hyperfine structure ($|A|$ = 1.45 mT) at g = 2.06 did not change (data not shown). As shown in Table 2, propargylamine inhibited pMMO activity. Propargylamine may inhibit pMMO activity by ketene formation in the active site, which binds the active site of pMMO irreversibly. The results suggest that the iron signals are attributed to the active site of pMMO, *i.e.* the active site of pMMO may contain an iron atom.

Zahn and co-workers reported the evidence for an iron center in the ammonia monooxygenase (AMO) from *Nitrosomonas europaea* (Zahn *et al.* 1996). AMO has been suggested to be homologous to the pMMO from *M. capsulatus* (Bath) and *M. trichosporium* OB3b (Senrau *et al.* 1995, Holmes *et al.* 1995). Treatment of the membranes containing AMO from *Nitrosomonas europaea* with nitrapyrin (2-chloro, 6-trichloromethylpyridine), which is a specific inhibitor of AMO, results in the increase in magnitude of a g = 6, high-spin ferric iron signal. In this study, when propargylamine was added to the EDTA-treated membranes, the pMMO activity and also the iron ESR signal decreased. The result is similar to the result of treatment of the membranes containing AMO from *Nitrosomonas europaea* with the specific inhibitor, *i.e.* the active site of AMO or pMMO has iron cluster.

Nitrosyl iron complexes in EDTA-treated membranes

Nitric oxide has been used to form ESR-active nitrosyl iron complexes of a non-heme iron center. Nitric oxide binds to the ferrous-containing proteins, leading to an ESR spectrum with a characteristic S = 3/2 spin due to the formation of a ferrous nitrosyl complex (Arciero *et al.* 1983, Zahn & DiSpirito 1996).

The inhibition of pMMO activity with propargylamine shows that iron is a component of pMMO from *M. trichosporium* OB3b. Thus, EDTA-treated membranes were treated with nitric oxide. And the ESR spectrum were compared with corresponding data of other non-heme iron proteins. Figure 5 (trace A) shows the ESR spectra of the EDTA-treated membranes from *M. trichosporium* OB3b at 5 K. The high-spin iron signal (g = 6.01) and the iron signal with g = 4.32 existed in the ESR spectrum of the EDTA-treated membranes (Fig. 5, trace A). As shown in Fig. 5 (trace B), the addition of nitric oxide under anaerobic conditions to the duroquinol-reduced membranes resulted in the decrease of intensity of the iron ESR signals (g = 4.32 and 6.01) and an intermediate spin, S = 3/2 (g = 4.01 and 4.11), with reduced non-heme iron centers appeared. Duroquinol can provide reducing equivalents for pMMO (Shiemke *et al.* 1995, Takeguchi *et al.* 1998b), leading to reduction of ferric

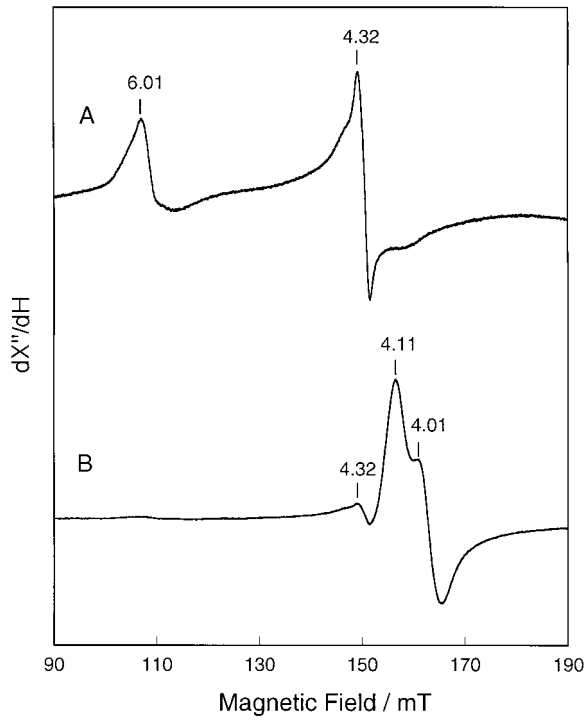

Figure 5. X-band ESR spectra of the EDTA-treated membranes treated by nitric oxide. (Trace A), the EDTA-treated membranes. (Trace B), the duroquinol-reduced membranes treated by nitric oxide under anaerobic condition. The spectra were recorded at 5 K with 2.0 mW of microwave power, modulation amplitude of 1.0 mT, modulation frequency of 100 kHz, time constant of 0.03 s, and gain of 125 (Trace A) and 40 (Trace B).

ion in the EDTA-treated membranes as shown in Fig. 3. The ESR spectrum of ferrous-nitric oxide derivative of pMMO from *M. capsulatus* (Bath) was previously reported (Zahn & DiSpirito 1996). Our results also show the ESR signal of a non-heme iron species with a spin state of S = 3/2 by the addition of nitric oxide to the reduced EDTA-treated membranes from *M. trichosporium* OB3b. The ferrous-nitric oxide derivative ESR spectrum of pMMO from *M. trichosporium* OB3b was similar to that of pMMO from *M. capsulatus* (Bath) and that of protocatechuate 4, 5-dioxygenase from *Pseudomonas testosteroni* (Arciero *et al.* 1983). Protocatechuate 4, 5-dioxygenase from *Pseudomonas testosteroni* has been purified to homogeneity and crystallized. The ferrous ion which is present in native protocatechuate 4, 5-dioxygenase reacts with nitric oxide to produce a species with a spin state of S = 3/2. The ferrous-nitric oxide derivative ESR spectrum of the dioxygenase indicates a mononuclear non-heme protein with *g* values around 4. The ESR experiments involving the ferrous nitrosyl complex in EDTA-treated membranes indicates that the iron in EDTA-treated membranes is a mononuclear non-heme iron.

Conclusion

The effect of metal ions, especially iron, on particulate methane monooxygenase (pMMO) activity was investigated against the EDTA-treated membranes. This spectrum of the EDTA-treated membranes shows that both the multiple hyperfine signal at g = 2.06 and the high-spin iron signal might be contained in the active site of pMMO from *M. trichosporium* OB3b, so that the activity of the EDTA-treated membrane remained 82% of the as-isolated membranes.

The activity in the EDTA-treated membranes was strongly influenced by the addition of metal ions. Among the metal ions, iron and copper ions stimulated the activity. These results indicate that both of copper and iron are needed for the activity of pMMO from *M. trichosporium* OB3b.

Like acetylene, propargylamine inhibited pMMO. The intensity of iron ESR signal decreased with an increasing of propargylamine concentration, indicating that the active site of pMMO contains iron. The ESR experiments involving the ferrous nitrosyl complex in EDTA-treated membranes indicated that the iron in EDTA-treated membranes is a mononuclear non-heme iron, *i.e.* the active site of pMMO may contain a mononuclear non-heme iron.

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