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# The role of copper in particulate methane monooxygenase from *Methylosinus trichosporium* OB3b

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**Abstract**

The effect of metal ions on particulate methane monooxygenase (pMMO) was studied. The pMMO activity in the membranes was partially inhibited by ethylenediaminetetraacetic acid (EDTA), but remained more than 70% of the as-isolated membranes. The activity of the EDTA-treated membranes was strongly influenced by the addition of metal ions. Among the metal ions, copper ion stimulated the activity, indicating that copper was needed for the activity. When duroquinol and dioxygen were introduced to the EDTA-treated membranes, the electron spin resonance signal of copper did not change, suggesting that the copper cluster did not play as an electron transport and may have another function, such as active site of pMMO or regulator of the activity. On the other hand, the iron signal ($g = 5.98$) decreased by the addition of duroquinol, dioxygen and acetylene, showing an iron atom is contained in the active site of pMMO. 

*Keywords:* Particulate methane monooxygenase; *Methylosinus trichosporium* OB3b; Copper; Iron; Acetylene

## 1. 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 [1]. 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 about pMMO, because of the instability. The role of copper in pMMO is not clarified yet. In this study, we investigated the effect of metal ions on the pMMO activity in the membranes

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

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treated by EDTA from *M. trichosporium* OB3b, and the role of copper is discussed.

## 2. Materials and methods

### *2.1. Materials*

Methane and propene were purchased from Fujiibussan. Duroquinone (tetramethyl-*p*-benzoquinone) and DNase I were obtained from Sigma–Aldrich Japan. Phenylmethylsulfonyl fluoride (PMSF) and 3-morpholinopropanesulfonic acid (MOPS) were obtained from Wako. 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 the University of Minnesota. *M. trichosporium* OB3b was cultivated as described by Fox et al. [2]. *M. trichosporium* OB3b was maintained on 3.5% agar plate with 1.25 $\mu$M $CuSO_4$. Large-scale growth was started in 200-ml baffle-walled shaking flask containing 27 ml medium with 5 $\mu$M $CuSO_4$, a 20% methane in 80% air atmosphere. These cultures were shaken at 30°C for approximately 4 days. Each culture was then transferred to a 500-ml baffle-walled shaking flask containing 100 ml medium with 10 $\mu$M $CuSO_4$ and maintained under the same conditions for 2 days. After this period, the cultures (260 ml to 390 ml total) were used to inoculate 2.3 l of medium containing 10–20 $\mu$M $CuSO_4$ in a 3-l fermentor (MBF-500 M, EYELA). The fermentor was maintained at 30°C with continuous stirring under 50% methane and 50% oxygen. After 5–7 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 ca. 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 in liquid $N_2$ and stored at −80°C.

### *2.3. 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) at 4°C under anaerobic conditions. Just prior to breaking the cells the suspension was supplemented with 300 $\mu$M $CuSO_4$ and with DNase I at the concentration of 10 $\mu$g/ml-suspension. During breaking the cells the suspension was supplemented with 1 mM PMSF in acetone. The broken cell was 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.

### *2.4. Treatment of membranes with EDTA*

The membrane fractions 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 $143\,000 \times g$ 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) and centrifuged at $143\,000 \times g$ for 90 min at 4°C. The supernatant was discarded, and membranes were resuspended by using homogenizer in 25 mM MOPS buffer (pH 7.0) and stored at −80°C under nitrogen.

### 2.5. pMMO assay

pMMO activity was measured by propene epoxidation [3]. The sample solution (2.5 ml) contains membrane fractions and duroquinol in 25 mM MOPS buffer (pH 7.0). The flask (ca. 10 ml) was sealed with a Teflon-sealed septa, and then incubated for 5 min at 30°C. The reaction was initiated by injecting 2.5 ml of propene into the flask with a gas-tight syringe. The produced propylene epoxide was measured by gas chromatography using a Sorbitol 25%-Gasport B column (4 m × 3 mm i.d., GL Sciences) attached to a Hitachi 263-30 gas chromatograph (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 [4].

### 2.6. Copper ion analysis

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

### 2.7. ESR spectroscopy

Electron spin resonance (ESR) spectra were recorded on a JEOL RE1X ESR spectrometer. The ESR samples were prepared by sealing 500 $\mu$l of membrane fractions under argon atmosphere in quartz ESR tubes. To prepare the samples, the membrane fractions were rapidly frozen in cooled *n*-heptane and liquid nitrogen.

Measurement of ESR intensity and spin concentration quantitation were carried out as follows. Cu–EDTA standard solutions in distilled water were prepared. The ESR spectra of these copper standards were recorded at 77 K with 1 mW of microwave power. The spectra were double-integrated, and a standard curve correlating the ESR intensity with copper concentration was generated. The level of ESR-detectable copper ions in the membranes was then inferred on basis of the ESR signal intensity and the standard curve.

## 3. Results and discussion

### 3.1. Effect of chelating agents on pMMO activity

As copper is contained in pMMO [5], the effect of metal chelating agents on the pMMO activity was investigated. The effects of chelating agents on pMMO activity are shown in Table 1. The pMMO activity in membrane fractions was inhibited by chelating agents, especially thiols. Though pMMO activity in the membranes was inhibited by EDTA, more than 70% activity remained even with high EDTA concentration.

The ESR spectrum of the EDTA-treated membranes was measured. The EDTA-treated membranes were prepared as described in Section 2. Fig. 1 shows the ESR spectra of the as-isolated membranes from *M. trichosporium* OB3b. The ESR spectra (Fig. 1, trace A) contained a weak high-spin iron signal ($g = 5.98$) and the type II copper signal ($g_{\|} = 2.24$, $A_{\|} =$ 18.4 mT, $g_{\perp} = 2.06$) observed in the 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 appearance of multiple hyperfine structure ($|A| = 1.45$ mT) at $g = 2.06$ (Fig. 1, trace C). But the EDTA-treated membrane resulted in the unchangeable intensity of the high-spin iron signal ($g = 5.98$). This spectrum of the EDTA-treated membranes shows that both the multiple hyperfine structure at $g = 2.06$ and the high-spin iron signal might be contained in the active site of pMMO from *M. trichosporium* OB3b, so

Table 1
Effect of chelating agents on propene epoxidation by membrane fractions from *M. trichosporium* OB3b

| Inhibitor | Concentration (mM) | Specific activity (nmol $min^{-1}$ mg $protein^{-1}$) | 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 |
| Thiourea | 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 |

that the activity of the EDTA-treated membrane remained more than 70% of the as-isolated membranes.

Both the copper contents of the as-isolated membranes and the EDTA-treated membranes by ESR method were in good agreement with copper ions analyzed by atomic absorption spectroscopy. The copper content (copper/protein ratio) of the EDTA-treated membrane was 54.3 $\mu$mol mg $protein^{-1}$ and that of the as-isolated membranes was 94.4 $\mu$mol mg $protein^{-1}$.

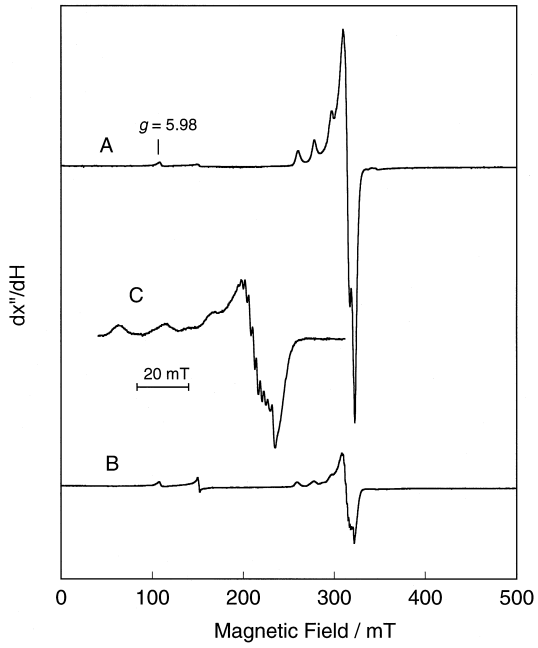


Fig. 1. X-band ESR spectrum of the EDTA-treated membrane fractions. (Trace A) The membrane fractions obtained from *M. trichosporium* OB3b. (Trace B, C) The membrane fractions treated by EDTA. The spectrum was recorded at 6.7 K with 0.2 mW of microwave power except for trace C (20 $\mu$W), modulation 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).

The spin concentration of the membrane was obtained by measurement of ESR intensity. The ESR spin quantitation on these as-isolated membranes indicated that approximately 27% of the total membrane-bound copper ions were ESR-active (Table 2). Similar results were obtained from the X-ray absorption studies of membranes from *Methylococcus capsulatus* (Bath) [6]. The X-ray absorption studies indicated that, in typical as-isolated preparations, approximately 70% of the total membrane-bound copper existed as Cu(I) [6]. Thus, the intensity of the ESR spectrum with the membranes was smaller than that obtained by the copper content of the membranes, so that the membrane contained high levels of copper ions with a significant portion of the copper ions existing as ESR-silent copper in the as-isolated membranes (70–80%), and the ESR-silent copper may be Cu(I).

As shown in Fig. 1 (trace B), the treatment of membranes by EDTA resulted in a decrease in the intensity of the copper ESR signal. The ESR spin quantitation on the EDTA-treated membranes indicated that 3% of the total membrane-bound copper ions were ESR-active (Table 2). Thus, the results indicated that the membrane-bound copper ions may exist in both Cu(II) and Cu(I) oxidation states, and EDTA

Table 2
Copper content of membrane fractions from *M. trichosporium* OB3b

| Sample | Specific activity (nmol $min^{-1}$ mg $protein^{-1}$) | Copper content ($\mu$mol g $protein^{-1}$) | Percentage of Cu(II) ions[a] |
|---|---|---|---|
| As-isolated membranes | 1.735 | 94.4 | 27% |
| EDTA-treated membranes | 1.431 | 54.4 | 3% |

[a] Percentage of Cu(II) ions is the content of ESR-active copper.

can chelate Cu(II) in the membrane-bound copper ions.

### 3.2. Effect of metal ions on pMMO activity in membranes treated by EDTA

The effect of metal ions against EDTA-treated membranes was studied. Addition of copper ions to the membranes treated with EDTA leads to reactivation of the enzyme (Fig. 2). The pMMO activity increased about two times and then decreased with increasing copper concentration. Maximum pMMO activity was obtained using 50 $\mu$M of copper concentration. This results indicate that copper ions are needed for the activity of pMMO from *M. trichosporium* OB3b and excess copper ions inhibit pMMO activity. The inhibition of pMMO activity by excess copper was observed in as-isolated membranes. When 300 $\mu$M Cu(II) was added to the membrane fractions with a pMMO activity of 4.07 nmol $min^{-1}$ mg $protein^{-1}$, the activity decreased to 1.24 nmol $min^{-1}$ mg $protein^{-1}$, and then was 30.4%. This phenomenon is also observed in other methanotrophs, but the reason for the inhibition of the pMMO activity by excess of copper is not clarified [5,7].

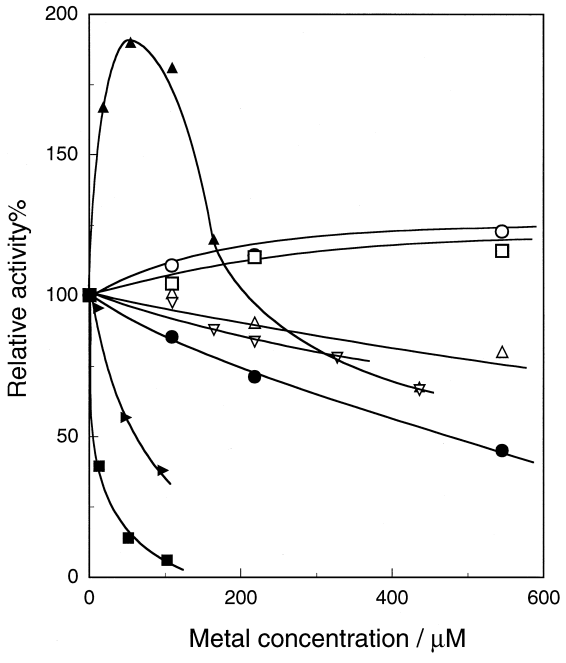


Fig. 2. The effect of metal ions on pMMO activity. The effect of exogenous copper (▲), cobalt (●), calcium (○), manganese (△), magnesium (□), iron (▼), nickel (►) and zinc (■) are shown for the membranes that was treated by 14.3 mM EDTA.

Addition of Ca(II) or Mg(II) to the EDTA-treated membrane resulted in a slight increase in pMMO activity. Magnesium was known as an activator of ammonia monooxygenase (AMO) from *Nitrosomonas europaea* [8]. AMO and pMMO have many enzymatic similarities and putative structural genes with strong sequence homology [9,10]. The effect of magnesium on pMMO activity may be the same reason on AMO activity.

In contrast, addition of Ni(II), Zn(II), Co(II), Mn(II) or Fe(II) inhibited pMMO activity. This phenomenon is also observed in other methanotrophs [5,7]. These metals may displace other metals such as copper from the active site of pMMO.

These results indicate that EDTA specifically chelates copper in pMMO, and copper ions reconstituted the copper site of pMMO subsequently.

### 3.3. Effect of substrates on ESR spectra of membranes and EDTA-treated membranes

As mentioned above, pMMO requires reducing agents for hydroxylation of methane. Quinols can provide reducing equivalents for pMMO

from *M. capsulatus* (Bath), substituting for NADH [11]. In this study, the activity of pMMO in membrane fractions was highest when duroquinol was used (data not shown). Although the solubility of the quinols is low in aqueous solution, duroquinol was more effective than NADH in membrane fractions. If the active site of pMMO consists of a copper cluster, the copper signal of ESR spectra of membranes may change when the substrates such as duroquinol and dioxygen were introduced to the membranes. Thus, the effect of duroquinol and dioxygen on ESR spectra of membranes was studied.

Fig. 3 (trace A) shows the ESR spectra of the EDTA-treated membrane fractions from *M. trichosporium* OB3b at 79 K. The multiple hyperfine structure ($|A| = 1.45$ mT) at $g = 2.06$ existed in the ESR spectrum of the EDTA-treated membranes (Fig. 3, trace A). As shown in Fig. 3, successive addition of excess amounts of duroquinol resulted in the unchangeable intensity of the copper ESR signal (Fig. 3, trace B). Upon exposure of this reduced sample to dioxygen, the copper ESR signal didn't change (Fig. 3, trace C). The results indicate that the copper cluster containing the multiple hyperfine structure ($|A| = 1.45$ mT) at $g = 2.06$ in EDTA-treated membranes does not play as an electron transfer and may have another function, such as active site of pMMO or regulator and stabilizer of pMMO activity.

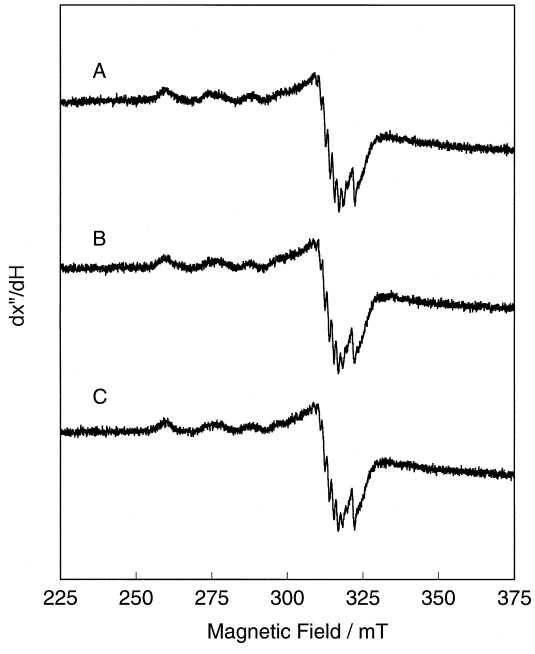


Fig. 3. X-band ESR spectrum of the EDTA-treated membrane fractions plus substrates. (Trace A), before adding duroquinol, i.e., the EDTA-treated membranes. (Trace B), after adding excess duroquinol under anaerobic condition. (Trace C), after reexposing the sample to oxygen. The spectrum was recorded at 79 K with 1 mW of microwave power, modulation amplitude of 0.5 mT, modulation frequency of 100 kHz, time constant of 0.03 s, and gain of 630.

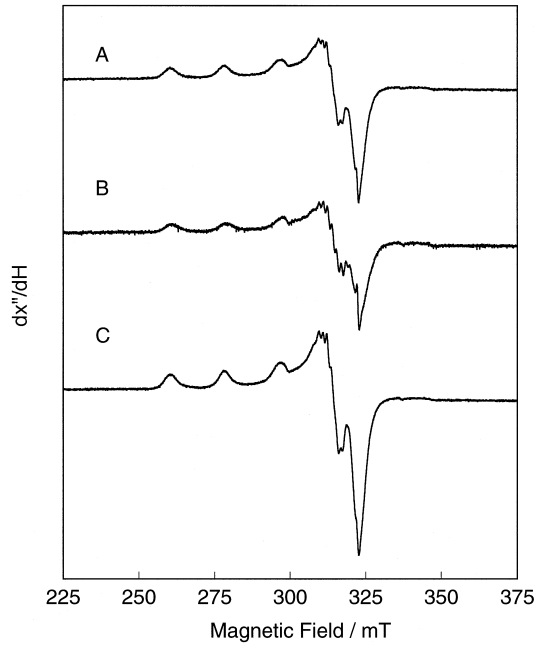


Fig. 4. X-band ESR spectrum of membrane fractions plus substrates. (Trace A), before adding duroquinol, i.e., as-isolated membranes. (Trace B), after adding excess duroquinol under anaerobic condition. (Trace C), after reexposing the sample to oxygen. The spectrum was recorded at 79 K with 1 mW of microwave power, modulation amplitude of 0.5 mT, modulation frequency of 100 kHz, time constant of 0.03 s, and gain of 125.

Fig. 4 (trace A), the same as shown in Fig. 1 (trace A), shows the ESR spectrum of membrane fractions from *M. trichosporium* OB3b at 79 K. As shown in Fig. 4, anaerobic and successive addition of excess amounts of duroquinol resulted in a decrease in the intensity of the

copper ESR signal and the appearance of multiple hyperfine structure at $g = 2.05$ (Fig. 4, trace B). Thus, the copper in membranes is reduced by duroquinol as a reductant of hydroxylation by pMMO. Upon exposure of this reduced sample to dioxygen, the copper ESR signal increased (Fig. 4, trace C). This result indicate that the copper cluster in membrane fractions is reduced and oxidized by duroquinol as an optimum reductant of pMMO and dioxygen as a substrate of pMMO, respectively. The results indicate that the copper cluster containing the type II copper signal ($g_{\|} = 2.24$, $A_{\|} = 18.4$ mT, $g_{\perp} = 2.06$) in membranes plays as an electron transfer. The same phenomenon was observed by Nguyen et al. when dithionite was used as reducing agent [12]. However, dithionite is not a reducing agent for hydroxylation of methane [11].

The results suggest that two types of copper cluster exists in membranes. One of the copper cluster containing type II copper signal in membranes plays as electron transfer. Another one containing the multiple hyperfine structure ($|A| = 1.45$ mT) at $g = 2.06$ in membranes plays as active site of pMMO or have another function, such as regulator and stabilizer of pMMO activity.

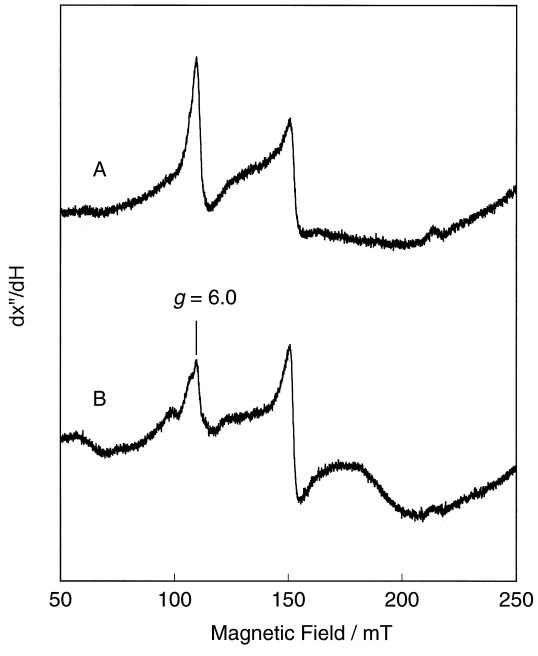


Fig. 5. X-band ESR spectrum of membrane fractions treated by acetylene. (Trace A), as-isolated membrane fractions. (Trace B), the membrane fraction treated by acetylene. The spectrum was recorded at 7.20 K with 0.2 mW of microwave power, modulation amplitude of 0.5 mT, modulation frequency of 100 kHz, time constant of 0.03 s, and gain of 500.

### *3.4. Effect of acetylene on the high-spin iron signal in membranes*

Fig. 5 (trace A) shows the ESR spectrum of the membrane fractions from *M. trichosporium* OB3b at 7.2 K. The ESR spectra (Fig. 5, trace A) contained a high-spin iron signal ($g = 5.98$). When duroquinol and acetylene were introduced to the membranes, the high-spin iron signal ($g = 5.98$) decreased (Fig. 5, trace B). Acetylene inhibited pMMO activity to bind a ketene in active site, as an intermediate of acetylene oxidation, to the active site of pMMO [13]. This result suggests that the high-spin iron signal ($g = 5.98$) is from the active site of pMMO, i.e., the active site of pMMO may contain an iron atom.

## 4. Conclusion

The effect of metal ions on particulate methane monooxygenase activity was investigated against the EDTA-treated membranes. The pMMO activity in membrane fractions was inhibited by chelating agents, especially thiols such as dithiothreitol and thiourea were strongly inhibited. When the membranes were inhibited by higher concentration of EDTA, the pMMO activity remained more than 70% 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 copper ion stimulated the activity. In contrast, addition of Ni(II), Zn(II), Co(II), Mn(II) or Fe(II) to the EDTA-treated membranes resulted in inhibition of pMMO activity. The results indicate that copper

is needed for the activity. However, by treatment of the EDTA-treated membranes with duroquinol and dioxygen, the copper signal did not change. The result suggests that the copper cluster does not play as an electron transport and may have another function, such as active site of pMMO or regulator of the activity.

By treatment of the membranes with duroquinol, dioxygen and acetylene the iron signal ($g = 5.98$) decreased, showing an iron atom is contained in the active site of pMMO.

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