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Redox behavior of copper in particulate methane monooxygenase from *Methylosinus trichosporium* OB3b

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The redox properties of the copper in particulate methane monooxygenase from *Methylosinus trichosporium* OB3b were investigated. The ESR spectrum of the pMMO-containing membranes from *M. trichosporium* OB3b indicated a typical type II copper (II) signal ($g_{\|}$ = 2.24, $A_{\|}$ = 18.4 mT, $g_{\perp}$ = 2.06, α^2 = 0.84). By anaerobic addition of excess amounts of duroquinol, an optimum reductant of pMMO, the ESR spectra indicated that the copper cluster in membranes was reduced and successively oxidized by dioxygen, a substrate of pMMO. The result suggests that the copper is the active site of pMMO or an electron carrier. During the titration, the intensity of the type II copper signal decreased with decreasing potential and the multiple hyperfine structure at *g* = 2.06 appeared clearly. Although the copper signal did not change by treatment of the EDTA-treated membranes with duroquinol and dioxygen, the copper signal intensity decreased with decreasing potential in the redox titration. These results suggest that some redox mediators play a role as an electron carrier between the active site and a reductant, and the presence of at least two types of copper sites in pMMO- containing membranes. On the basis of the ESR spectra of the EDTA-treated membranes and the as-isolated membranes, it is concluded that one type of the copper sites functions as the active site of pMMO (A-site), and the other type of copper sites plays a role as an electron carrier (E-site).

Keywords: particulate methane monooxygenase, *Methylosinus trichosporium* OB3b, redox titration

Abbreviations: MMO, methane monooxygenase; pMMO, particulate methane monooxygenase; sMMO, soluble methane 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 about pMMO, because of the instability. Recently, pMMO from *Methylococcus capsulatus* (Bath) and *M. trichosporium* OB3b have been purified and it is reported that the enzyme may contain both copper and iron (Zahn & Dispirito 1996, Takeguchi *et al*. 1998a). It was proposed that the active site of pMMO from *M. capsulatus* (Bath) was a copper cluster on the basis of the dependence of pMMO activity with copper concentration and the ESR spectrum (Nguyen *et al*. 1994). Nguyen and

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co-workers reported the presence of at least two types of copper ions in pMMO from *M. capsulatus* (Bath) (Nguyen *et al.* 1996). Whereas, the role of copper in pMMO is not clarified yet, in this paper, we hope to describe the redox behavior of the copper in membranes from *M. trichosporium* OB3b, and the role of copper 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 and quinhydrone were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Resorufin was purchased from Nacalai Tesque, Inc. (Kyoto, 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.

ESR Spectroscopy

X-band ESR spectra were recorded on a JEOL RE1X ESR spectrometer (JEOL, Tokyo, Japan) at liquid nitrogen temperature (77K). 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.

The α^2-value evaluates unpaired-electron density on the *d*-orbital of the copper (II) ion, and was calculated from ESR parameters by the following equation of Kivelson and Neiman (Kivelson & Neiman 1961).

$$\alpha^2 = A_{\parallel} / P + (g_{\parallel} - 2.0023) + 3 / 7 (g_{\perp} - 2.0023) + 0.04$$

$$P = 360 \times 10^{-4} \text{ cm}^{-1}$$

Redox Titration

The redox titrations were 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 (16μM 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 gastight 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

Effect of substrates on ESR spectrum of membranes

Figure 1 (*trace* A) shows the X-band ESR spectrum of membranes from *M. trichosporium* OB3b. This ESR spectrum indicated a typical type II copper (II) signal ($g_{\parallel} = 2.24$, $A_{\parallel} = 18.4$ mT, $g_{\perp} = 2.06$, $\alpha^2 = 0.84$). The correlation between $g_{\parallel}$ -values and $A_{\parallel}$ -values of the copper proteins and copper complexes is shown in Fig. 2 (Peisach & Blumberg 1974, Yokoi & Addison 1977). As shown in Fig. 2, the ESR parameters of membranes from *M. trichosporium* OB3b

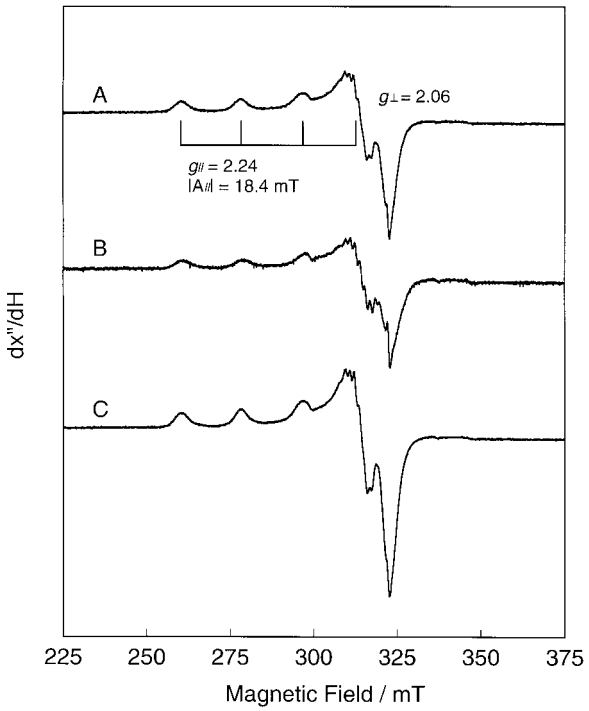


Figure 1. X-band ESR spectrum of membranes plus substrates. (*trace* A), before adding duroquinol, i.e. as-isolated membranes. (*trace* B), after adding excess duroquinol under anaerobic condition. (*trace* C), after reexposing the sample to oxygen. The spectrum was recorded at 79K with 1.0 mW of microwave 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.

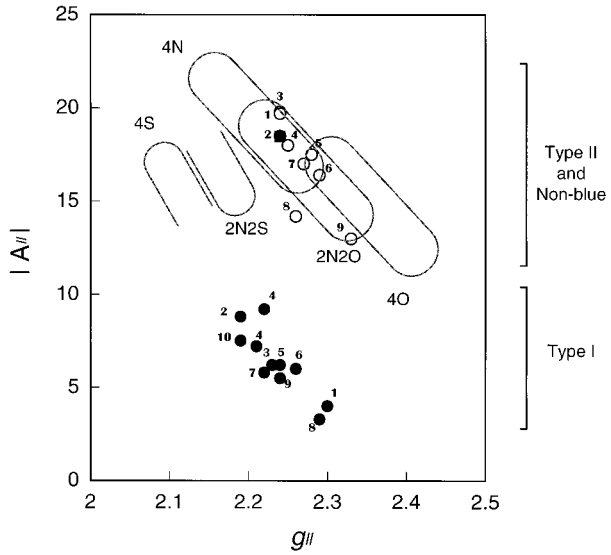


Figure 2. The relationship between gll and lAlll for copper proteins. Type I copper (○), type II copper (●), and the membranes from *M. trichosporium* OB3b (■). The regions set off by dashed lines are those of copper model complexes shown in Reference (Peisach & Blumberg 1974). (Type I) 1, laccase (lacquer); 2, laccase (fungus); 3, ascorbate oxidase (cucumber); 4, ceruloplasmin; 5, plastocyanin; 6, azurin; 7, prantacyanin; 8, stellacyanin; 9, pseudoazurin; 10, nitrate reductase, (Type II) 1, laccase (lacquer); 2, laccase (fungus); 3, ascorbate oxidase (cucumber); 4, ceruloplasmin; 5, galactose oxidase; 6, amine oxidase; 7, dopamin hydroxylase; 8, superoxide dismutase; 9, nitrate reductase.

are very similar to the one found for a type II copper in fungal laccase, suggesting that four nitrogen ligand atoms coordinate with the copper (II) ion. The α_2-value of the membranes related closely to a square- planar copper complexes (Sawada *et al.* 1996). Therefore, the copper in the membranes is presumed to have a square-planar configuration coordinated by four nitrogen ligand atoms. Nguyen and co-workers reported that anaerobic titration of highly oxidized membranes from *M. capsulatus* (Bath) by dithionite leaded 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 Fig. 1, anaerobic addition of excess amounts of duroquinol resulted in a decrease in the intensity of the copper (II) ESR signal by 53.8% of no addition, and successive addition of duroquinol resulted the appearance of the multiple hyperfine structure at g = 2.06 (Fig. 1, *trace* B). Upon exposure of this reduced sample to dioxygen, the copper (II) ESR signal increased to 137% of no addition (Fig. 1, *trace* C). The ESR spectra indicated that copper in membranes was reduced by duroquinol as an optimum reductant of pMMO and oxidized by dioxygen as a substrate of pMMO. The result suggests that the copper is contained in the active site of pMMO or an electron carrier.

Redox behavior of copper in membranes

ESR spectra of the membranes from *M. trichosporium* OB3b observed during the reductive titrations are shown in Fig. 3. During the titration, the intensity of the characteristic type II copper signal decreased with decreasing potential. The g = 2.01 signal can be assigned as radical signals generated by the reduced mediators. During the course of reductive titration, the multiple hyperfine structure at g = 2.06 appeared at +188 mV. The shape of the ESR signal at g = 2.06 is very similar to that of the duroquinol-reduced membranes as shown in Fig. 1

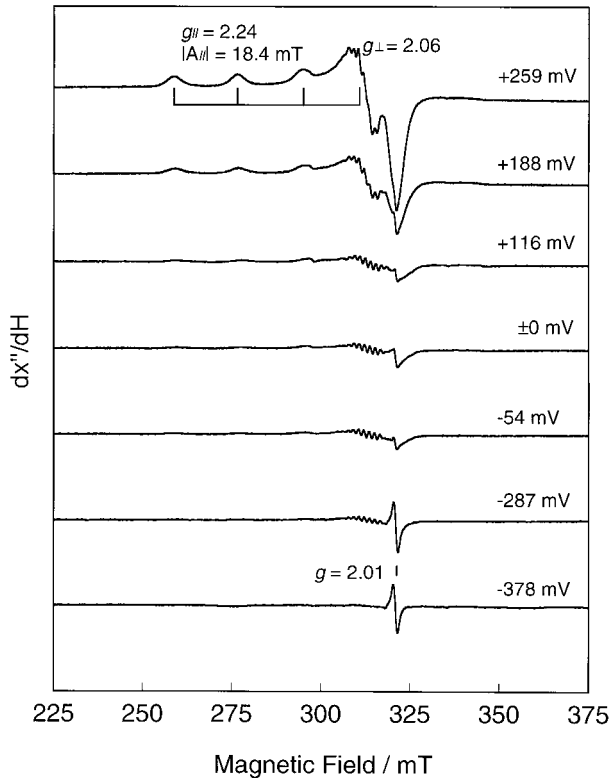


Figure 3. X-band ESR spectrum of the membranes during reductive titration. The potentials at which the samples were poised are indicated. The spectrum was recorded at 79 K with 1.0 mW of microwave power, modulation amplitude of 0.5 mT, modulation frequency of 100 kHz, time constant of 0.03 s, and gain of 63. The microwave frequency was 9.006 Ghz.

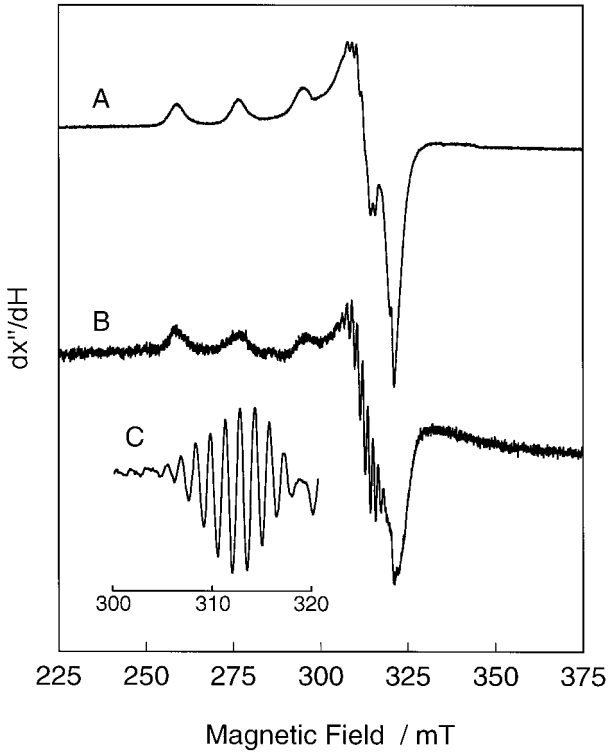


Figure 4. X-band ESR spectrum of the EDTA-treated membranes. (*trace* A), The membranes obtained from *M. trichosporium* OB3b. (*trace* B), The membranes treated by EDTA. The spectrum was recorded at 77 K with 1.0 mW of microwave power, modulation amplitude of 0.5 mT, modulation frequency of 100 kHz, time constant of 0.03 s, and gain of 79 (*trace* A) and 500 (*trace* B). The microwave frequency was 8.991 GHz. (*trace* C), second derivative of the *trace* B at g=2.06.

(*trace* B). Thus, anaerobic and successive addition of dithionite resulted in a decrease in the intensity of the copper (II) ESR signal and the appearance of the multiple hyperfine structure at g = 2.06. The intensity of the multiple hyperfine structure at g = 2.06 decreased with decreasing redox potential and vanished completely below –378 mV.

Effect of substrates on ESR spectrum of EDTA-treated membranes

In our previous study of the effect of metal chelating agents on the pMMO activity, the pMMO activity in membranes was partially inhibited by EDTA, because of 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 more than 70% of the as-isolated. Figure 4 shows the ESR spectra of the as-isolated and EDTA-treated membranes from *M. trichosporium* OB3b. The treatment of the membranes by EDTA resulted in a decrease in the intensity of the copper (II) ESR signal (Fig. 4, *trace* B) and then in the appearance of multiple hyperfine structure (|A| = 1.45 mT) at g = 2.06 (Fig. 4, *trace* B, C). This spectrum of the EDTA-treated membranes shows that the multiple hyperfine structure at g = 2.06 might be related to the active site of pMMO from *M. trichosporium* OB3b, since the activity of the EDTA-treated membrane remained more than 70% of the as-isolated membranes. Nguyen and co-workers reported that this hyperfine structure indicated a splitting arising from the coupling of one unpaired electron spin to three equivalent I = 3/2 nuclear spins, i.e. it may have origin in a trinuclear copper cluster (Nguyen *et al.* 1994). As shown in Fig. 1 (*trace* B) and Fig. 4 (*trace* B), the multiple hyperfine structure (|A| = 1.45 mT) at g = 2.06 is observed

in the ESR spectrum of pMMO from *M. trichosporium* OB3b. In these spectra 10 nuclear hyperfine lines are observed, and the spectral intensity distribution of these hyperfine lines was in approximate ratio of 1:3:6:10:12:12:10:6:3:1. These results support the hypothesis of Nguyen *et al.* that the hyperfine splitting pattern has origins in a two-electron reduced trinuclear copper cluster. As mentioned above, anaerobic and successive addition of excess amounts of duroquinol or dioxygen to as-isolated membranes resulted in a redox of the copper (II) ESR signal and the appearance of the multiple hyperfine structure at g = 2.06 (Fig. 1). Thus, the effect of substrates such as duroquinol and dioxygen on the ESR signal of EDTA-treated membranes were investigated. As shown in Fig. 5, successive addition of excess amounts of duroquinol to EDTA-treated membranes resulted in no change of the intensity of the copper ESR signal (Fig. 5, *trace* B). Upon exposure of this reduced sample to dioxygen, the copper ESR signal again did not change (Fig. 5, *trace* C). These results indicate that the copper cluster containing the multiple hyperfine structure at g = 2.06 in EDTA-treated membranes does not play a role as an electron carrier and may have another function such as active site of pMMO.

Redox behavior of copper in EDTA-treated membranes

The EDTA-treated membranes, which may contain the active site of pMMO, were used for redox titration of the copper cluster. The result is shown in Fig. 6. During the titration, the intensity of the type II copper signal decreased with decreasing potential. The appearance at –296 mV of the g = 2.01 signal which can be assigned as due to radicals signals generated by the reduced mediators. The multiple hyperfine structure at g=2.06 appeared clearly and decreased with decreasing redox potential. The results were similar to that of the as-isolated membranes. Although the copper signal did not

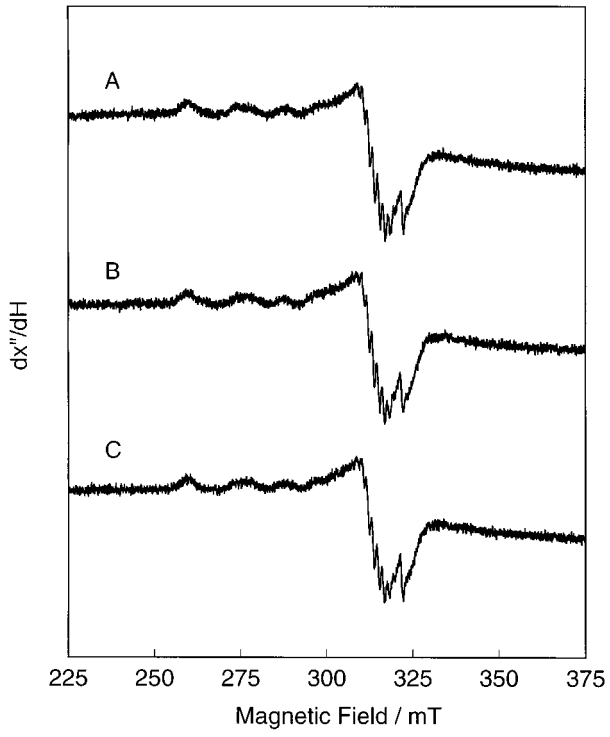


Figure 5. X-band ESR spectrum of the EDTA-treated membranes plus substrates. (*trace* A), before adding duroquinol, i.e. the EDTA-treated membranes. (*trace* B), after adding excess duroquinol under anaerobic condition. (*trace* C), after reexposing the sample to oxygen. The spectrum was recorded at 79K 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. The microwave frequency was 8.990 GHz.

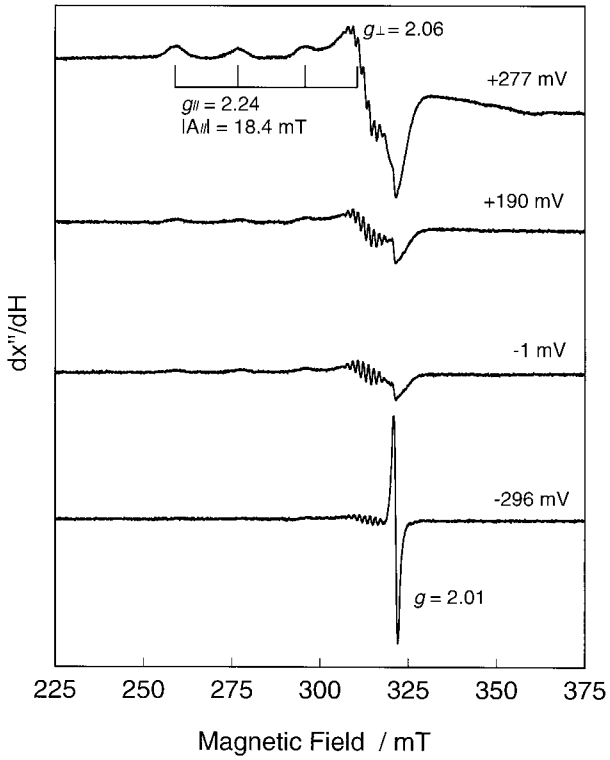
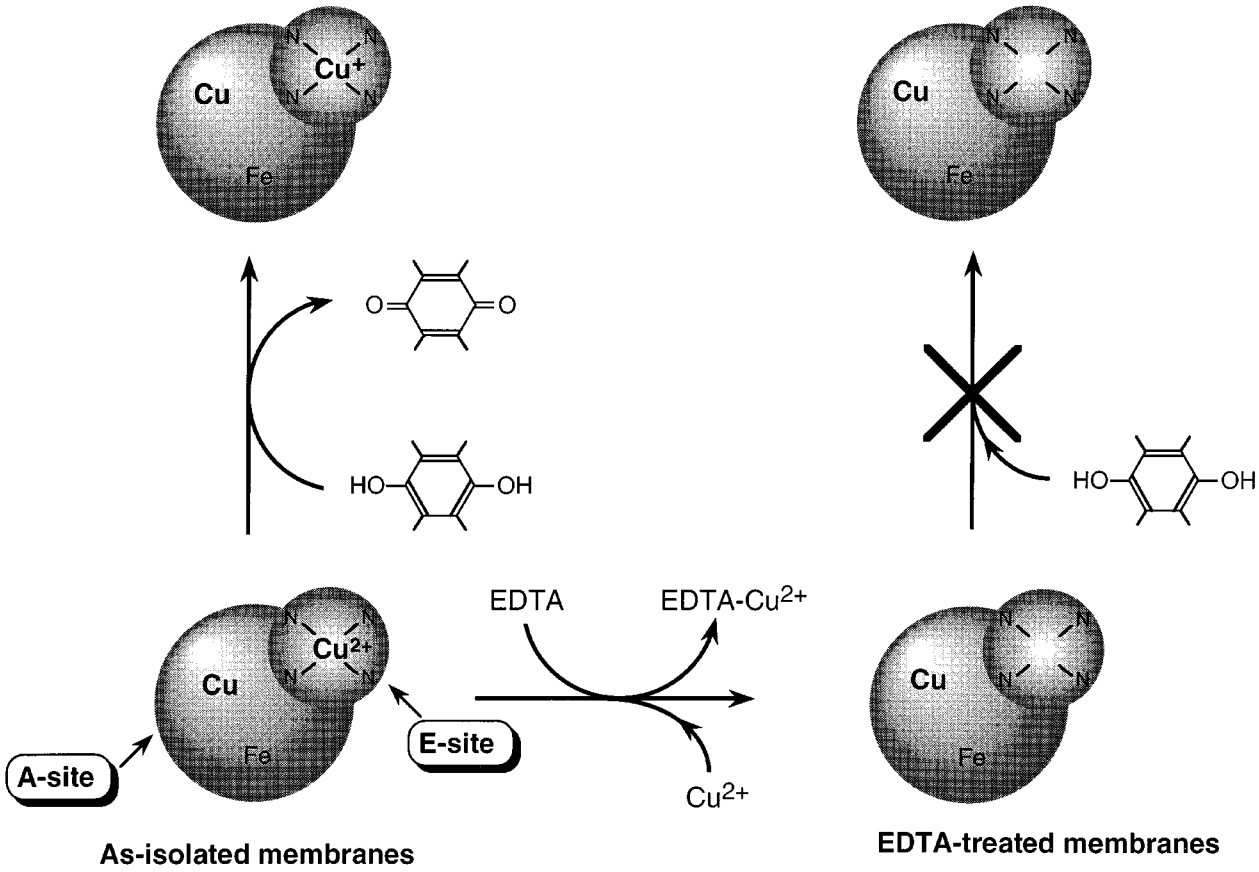


Figure 6. X-band ESR spectrum of the EDTA-treated membranes during reductive titration. The potentials at which the samples were poised are indicated. The spectrum was recorded at 79 K with 1.0 mW of microwave power, modulation amplitude of 0.5 mT, modulation frequency of 100 kHz, time constant of 0.03 s, and gain of 200. The microwave frequency was 9.011 GHz.



Scheme 1. Hypothetical scheme of two types of copper clusters in pMMO-containing membranes

change by treatment of the EDTA-treated membranes with duroquinol and dioxygen, the copper signal decreased with decreasing potential in the redox titration. This results suggest that some redox mediators play a role as an electron carrier between the active site and a reductant.

Mechanism: two types of copper sites in pMMO-containing membranes

These results suggest the presence of at least two types of copper sites in the pMMO-containing membranes from *M. trichosporium* OB3b. The following mechanism is proposed: one type of copper sites functions primarily as the active site of pMMO (A-site), on the basis of the ESR spectrum of the EDTA-treated membranes, i.e. the multiple hyperfine structure ($|A| = 1.45$ mT) at $g = 2.06$. The other type of copper sites performs as an electron carrier (E-site), on the basis of the ESR spectrum of the as-isolated membranes, i.e. the type II copper (II) signal ($g_{\|} = 2.24$, $A_{\|} = 18.4$ mT, $g_{\perp} = 2.06$, $\alpha^2 = 0.84$). As shown in Scheme 1, the as-isolated membranes contain two types of copper sites (A-site and E-site). The ESR spectra of the as-isolated membranes indicated typical type II copper (II) signal. The type II copper signal, which may indicate E-site, was reduced by duroquinol and reductive titration in anaerobic condition, and was re-oxidized by dioxygen. Thus, the E-sites may have a function as an electron carrier between reductant and active site of pMMO. The ESR parameters of the E-site are very similar to the those found for a type II copper in fungal laccase, suggesting that four nitrogen ligand atoms coordinate the copper (II) ion. In addition the α^2-value of the membranes related closely to a square-planar copper complexes. Together this indicated that the copper in membrane fractions is presumed to have a square-planar configuration coordinated by four nitrogen ligand atoms. When the as-isolated membranes were treated by EDTA, the copper content in the EDTA-treated membranes decreased to 58% of the as-isolated membranes, and the ESR spectra indicated the presence of the multiple hyperfine structure at $g = 2.06$. The multiple hyperfine structure may be part of the

active site of pMMO from *M. trichosporium* OB3b, i.e. The A-site, since the activity of the EDTA-treated membrane remained more than 70% of the as-isolated membranes. The multiple hyperfine structure at $g = 2.06$, which may indicate A-site, was not affected by duroquinol, indicating that the EDTA-treated membranes do not contain the E-site. However, the multiple hyperfine structure was reduced by reductive titration with redox mediators. The result suggests that these redox mediators may play as electron carrier such as E-site.

Acknowledgements

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