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Journal of Molecular Catalysis A: Chemical 132 (1998) 145–153

JOURNAL OF MOLECULAR CATALYSIS A: CHEMICAL

# Purification and properties of particulate methane monooxygenase from *Methylosinus trichosporium* OB3b

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Received 16 October 1997; accepted 17 November 1997

**Abstract**

Particulate methane monooxygenase (pMMO) from *Methylosinus trichosporium* OB3b has been solubilized with *n*-dodecyl-β-D-maltoside and purified by chromatographic techniques. The molecular weight was estimated to 326 kDa consisting of two subunits with molecular masses of 25 and 41 kDa. The enzyme contained 0.9 iron atom and 12.8 copper atoms/molecule. The electron spin resonance (ESR) spectra of the enzyme showed a type II copper ($g_{\|} = 2.24$, $A_{\|} = 18.4$ mT, $g_{\perp} = 2.06$) and a weak high-spin iron signal ($g = 5.98$). By treatment of pMMO with duroquinol, dioxygen and acetylene, the iron signal disappeared, showing an iron atom is contained in 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 according to the following equation:

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

The copper concentration during the growth of *Methylosinus trichosporium* OB3b controls the expression type of MMO, soluble or membrane-bound [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 about pMMO. Recently, purification of pMMO from *Methylococcus capsulatus* (Bath) has been reported and the enzyme may contain both copper and iron [2]. In this study, pMMO from *M. trichosporium* OB3b was purified and characterized, and some enzymatic properties of the enzyme were described and the active site of pMMO is discussed.

## 2. Materials and methods

### 2.1. Materials

All the chemicals were of the highest grade available and were used without further purifi-

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1381-1169/98/$19.00 
*PII* S1381-1169(97)00275-6

cation. Methane, propene and acetylene were purchased from Fujiibussan. Duroquinone (tetramethyl-*p*-benzoquinone), DNase I (from bovine pancreases) and molecular weight markers for gel filtration (MW-GF-1000 kit) were obtained from Sigma Chemical. Molecular weight standards for SDS–PAGE (low range) were obtained from Bio-Rad. Phenylmethylsulfonyl fluoride (PMSF), *n*-octyl-$\beta$-D-glucoside, *n*-dodecyl-$\beta$-D-maltoside, sucrose monocaprate (SM-1080), sucrose monolaurate (SM-1280) and 3-morpholinopropanesulfonic acid (MOPS) were obtained from Wako Pure Chemical Industries. Metal (copper and iron) standard solution for atomic absorption spectrometry and the other chemicals were purchased from Kanto Chemical.

### 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 was cultivated as described by Fox et al. [3]. *M. trichosporium* OB3b was maintained on 3.5% agar plate with 1.25 $\mu$M $Cu(SO_4)_2$. Large-scale growth was carried out in 200-ml baffle-walled shaking flask containing 27 ml medium with 5 $\mu$M $Cu(SO_4)_2$, a 20% methane in 80% air atmosphere. These cultures were shaken at 30°C for 4 days. Each culture was then transferred to a 500-ml baffle-walled shaking flask containing 100 ml medium with 10 $\mu$M $Cu(SO_4)_2$ and maintained under the same conditions for 2 days. After this period, the cultures were used to inoculate 2.3 l of medium containing 10 $\mu$M $Cu(SO_4)_2$ in a 3-l fermentor (MBF-500M, EYELA). The fermentor was maintained at 30°C with continuous stirring and 50% methane and 50% oxygen were introduced. 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/l. The bacterial pellet was washed with 25 mM MOPS buffer (pH 7.0), and resuspended in the same buffer. The resuspended cells were rapidly frozen in liquid $N_2$ and stored at $-80$°C.

### 2.3. pMMO assay

The pMMO activity was measured by propene epoxidation [4]. The sample solution (0.5 ml) contained 10 mM duroquinol as a solid in 25 mM MOPS buffer (pH 7.0). The vial (ca. 3 ml) was sealed with a Teflon-sealed septa, and then incubated at 30°C for 5 min. The reaction was initiated by injecting 0.5 ml of propene into the flask with a gas-tight syringe. The produced propylene oxide was measured by gas chromatography using a Sorbitol 25%-Gasport B column (4 m $\times$ 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]. Specific activity was obtained by dividing the activity by the total amount of protein in the sample determined by the method of Lowry et al. [5].

### 2.4. Preparation of duroquinol

The methods for reduction of duroquinone to duroquinol was performed by a modification of the method described by Shiemke et al. [6]. All procedures were performed under anaerobic conditions by adding excess sodium dithionite and under nitrogen atmosphere.

### 2.5. 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 anaerobically. Just prior to break the cells, the suspension was supplemented with 300 $\mu$M $Cu(SO_4)_2$ and with DNase I (10 $\mu$g/ml suspension). During breaking the cells, the suspension was supplemented with 1 mM PMSF in acetone. The broken cells were cen-

trifuged 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), and the membrane fractions were stored at −80°C under nitrogen.

### 2.6. Purification of pMMO

Purification of pMMO from *M. trichosporium* OB3b was performed by a modification of the method described by Zahn and DiSpirito [7]. All procedures were performed anaerobic conditions at 4°C. The membrane fractions (294 mg protein) was transferred into a 50-ml Erlenmeyer flask. The flask was sealed with a white-rubber septa, and then incubated under purging nitrogen gas for 20 min in the dark. After incubation, 10% (w/v) solution of *n*-dodecyl-β-D-maltoside was added to a final concentration, 0.92 mg detergent/mg protein, with a gas-tight syringe. The solution was stirred at 4°C under purging nitrogen gas for 45 min and then centrifuged at $140\,400 \times g$ for 90 min at 4°C. The detergent-solubilized membrane fraction was diluted to 6 mg detergent/ml to add deoxygenated 25 mM MOPS buffer (pH 7.0) and loaded on to a DEAE Sepharose Fast Flow column ($10 \times 110$ mm, Pharmacia) and at a flow rate of 76 cm/h washed with deoxygenated 25 mM MOPS buffer (pH 7.0). The pMMO did not bind to the column, and it eluted in the flowthrough fractions. The flowthrough fractions were loaded on to a Phenyl Sepharose 6 Fast Flow, low sub ($10 \times 90$ mm, Pharmacia) equilibrated with deoxygenated 25 mM MOPS buffer (pH 7.0). The column was washed at a flow rate of 15 cm/h with 1 column volume of deoxygenated 25 mM MOPS buffer (pH 7.0) containing 0.01% detergent. Then the enzyme was eluted with a linear gradient of detergent concentrations from 0.01 to 0.9%. The isolated pMMO was rapidly frozen in liquid $N_2$ and was stored at −80°C under nitrogen.

Table 1
Effect of detergent for the solubilization of pMMO from membranes

| Detergent | Activity (%)[a] | |
|---|---|---|
| | Solubilized | Residual |
| *n*-Octyl-β-D-glucoside | 0 | 0 |
| *n*-Dodecyl-β-D-maltoside | 5.7 | 2.4 |
| Sucrose monolaurate[b] | 3.4 | 2.0 |
| Sucrose monocaprate[b] | < 2.6 | 2.1 |

[a] The activity was assayed as described in Section 2. The rate of membrane fractions, which corresponds to 100%, was 2.04 nmol/min per mg protein.
[b] These detergents contain about 20% di- or triester.

### 2.7. Determination of molecular weight by gel filtration

Gel filtration for determination of molecular weight of pMMO was performed using Sephacryl S-300 High Resolution ($15 \times 984$ mm, Pharmacia). The column was equilibrated with 25 mM MOPS buffer (pH 7.0) containing 0.01% *n*-dodecyl-β-D-maltoside and 0.1 M NaCl. Molecular weight determination of pMMO was made by comparing the ratio of $V_e/V_0$ for the pMMO in the $V_e/V_0$ of protein

Table 2
Effect of detergent concentration for the solubilization of pMMO from membranes

| Detergent-to-protein[a] (mg detergent/mg protein) | Activity%[b] | | Total activity (%)[b] |
|---|---|---|---|
| | Solubilized | Residual | |
| 0.46 | 0.99 | 14.5 | 15.5 |
| 0.92 | 7.60 | 1.49 | 9.1 |
| 1.84 | 1.48 | N.D.[c] | 1.5 |

[a] Detergent is *n*-dodecy-β-D-maltoside.
[b] The activity was assayed as described in Section 2. The rate of membrane fractions, which corresponds to 100%, was 2.80 nmol/min per mg protein.
[c] N.D. = Not detected.

Table 3
Purification of pMMO form *M. trichosporium* OB3b

| Step | Total protein (mg) | Total activity (nmol/min) | Specific activity (nmol/min per mg) | Purification (-fold) |
|---|---|---|---|---|
| Whole cells[a] | – | 235 000 | – | – |
| Membrane fractions | 294 | 668 | 2.27 | – |
| Detergent extract[b] | 142 | 34.6 | 0.243 | 1.00 |
| DEAE sepharose fast flow | 67.1 | 14.6 | 0.218 | 0.90 |
| Phenyl sepharose fast flow | 12.2 | 5.73 | 0.469 | 2.15 |

[a] Formate (14 mM) was used as the pMMO reductant.
[b] The membrane fractions were solubilized with *n*-dodecyl-β-D-maltoside.

standards of known molecular weight ($V_e$ is the elution volume and $V_0$ is the void volume). The void volume of a given column is based on the volume of effluent required for the elution of Blue Dextran. A calibration curve can then be prepared by plotting the logarithms of the known molecular weights of protein standards vs. their respective $V_e/V_0$ values. Cytochrome c ($M_r$ 12 400), carbonic anhydrase ($M_r$ 29 000), bovine serum albumin ($M_r$ 66 000), alcohol dehydrogenase ($M_r$ 150 000), β-amylase ($M_r$ 200 000), apoferritin ($M_r$ 443 000) and thyroglobulin ($M_r$ 669 000) were used as the reference proteins.

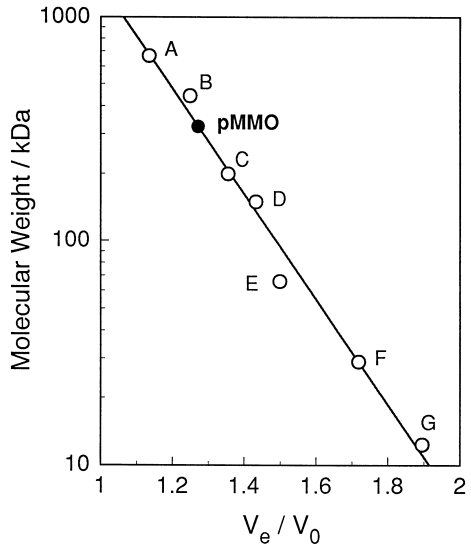



Fig. 1. Estimation of molecular weight of pMMO from *M. trichosporium* OB3b by gel filtration on Sephacryl S-300 H.R. Column size, 1.5×98 cm; flow rate, 12 cm/h; Molecular weight standard, A; thyroglobulin ($M_r$ 669 000), B; apoferritin ($M_r$ 443 000), C; β-amylase ($M_r$ 200 000), D; alcohol dehydrogenase ($M_r$ 150 000), E; bovine serum albumin ($M_r$ 66 000), F; carbonic anhydrase ($M_r$ 29 000), G; cytochrome c ($M_r$ 12 400).

### 2.8. SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis (SDS–PAGE) was performed by the method of Laemmli [8]. The separating gels contained 12% T and 2.6% C, and the stacking gels contained

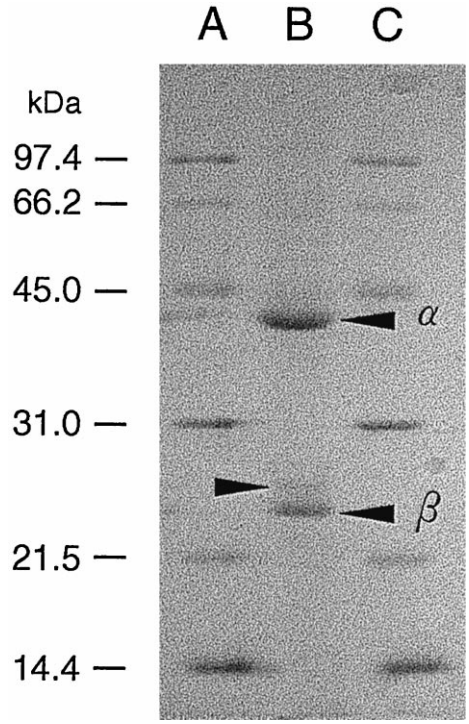



Fig. 2. SDS–polyacrylamide gel electrophoresis of isolated pMMO from *M. trichosporium* OB3b. Electrophoresis was performed on a 12% T, 2.6% C slab gel according to the Laemmli method and the gel was stained with Coomassie brilliant blue R-250. The two subunits of isolate pMMO are marked by α and β in lane B. Bio-Rad low range molecular weight standards are shown in lanes A and C.

3.0% T and 20% C. Phosphorylase b ($M_r$ 97 400), serum albumin ($M_r$ 66 200), ovalbumin ($M_r$ 45 000), carbonic anhydrase ($M_r$ 31 000), trypsin inhibitor ($M_r$ 21 500), lysozyme ($M_r$ 14 400) were used as the reference proteins.

### 2.9. Metal ion analysis

Metal ion (copper and iron) analysis was performed by inductively coupled plasma atomic emission spectroscopy (ICP–AES) on a SPS1500VR (Seiko Instruments). The metal concentration of the samples was determined relative to standard solutions. 25 mM MOPS buffer (pH 7.0) containing 0.01% *n*-dodecyl-β-D-maltoside was used as a metal-free control.

### 2.10. Electron spin resonance (ESR) spectroscopy

ESR spectra were recorded on a JEOL RE1X ESR spectrometer. The ESR samples were prepared by sealing 500 μl of isolated pMMO under an atmosphere of argon in quartz ESR tubes. Then, the samples were rapidly frozen in cooled *n*-heptane and liquid nitrogen.

## 3. Results and discussion

### 3.1. Purification of pMMO from M. trichosporium OB3b

After the cultivation, the bacterial sonicate was centrifuged and 91.6% of MMO activity

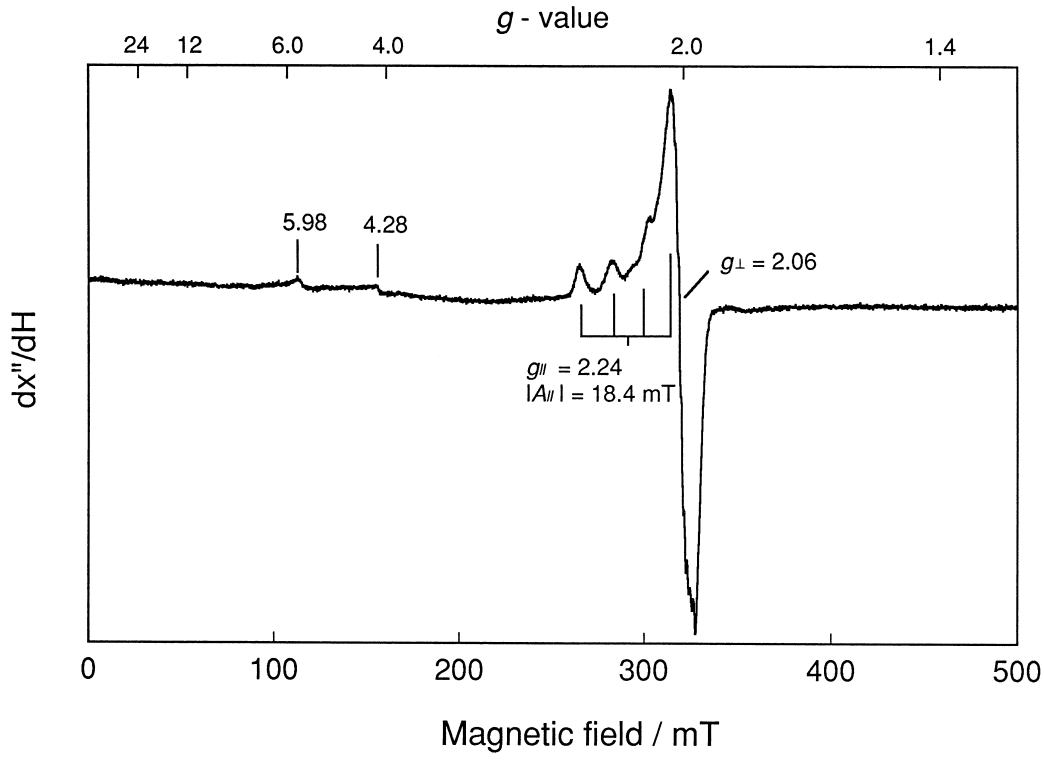



Fig. 3. X-band ESR spectra of isolated pMMO from *M. trichosporium* OB3b. The protein concentration was 2.6 mg protein/ml in 25 mM MOPS (pH 7.0) buffer containing 0.01% *n*-dodecyl-β-D-maltoside. The spectra was recorded at 7.10 K with 0.20 mW of microwave power, modulation amplitude of 0.5 mT, modulation frequency of 100 kHz, time constant of 0.03 s, and gain of 125. The microwave frequency was 8.985 GHz.

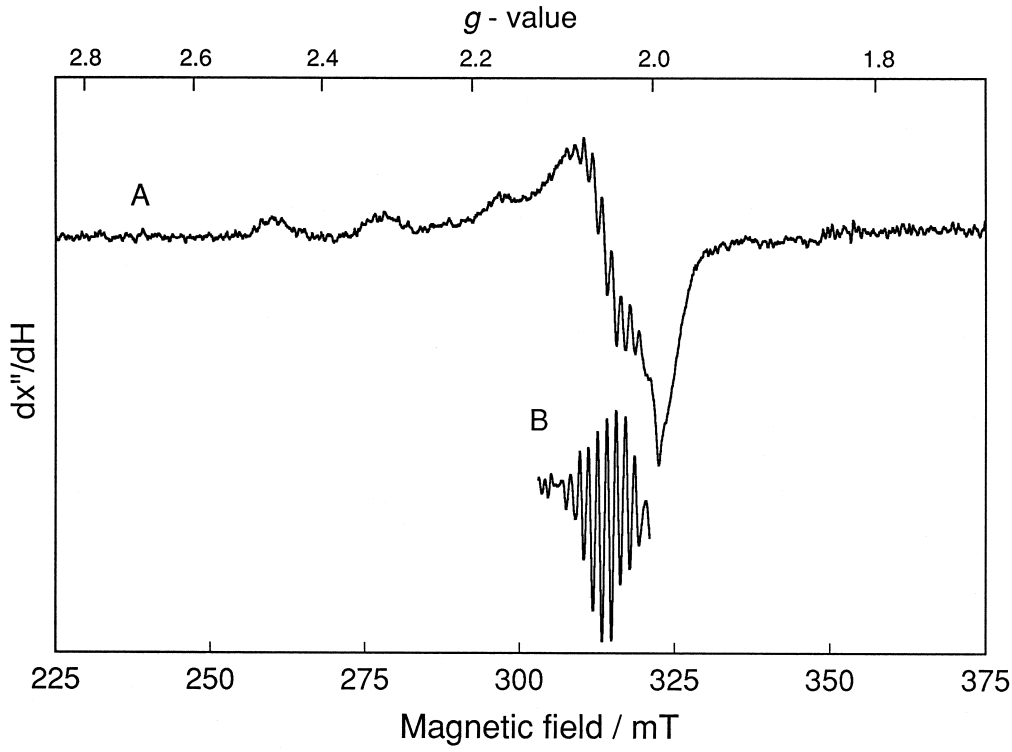



Fig. 4. X-band ESR spectra of isolated pMMO from *M. trichosporium* OB3b. The protein concentration was 2.6 mg protein/ml in 25 mM MOPS (pH 7.0) buffer containing 0.01% *n*-dodecyl-*β*-D-maltoside. The spectra A was recorded at 78.1 K with 1.00 mW of microwave power, modulation amplitude of 0.5 mT, modulation frequency of 100 kHz, time constant of 0.03 s, and gain of 1000. The microwave frequency was 9.000 GHz. B, second derivative of the absorption at $g = 2.06$.

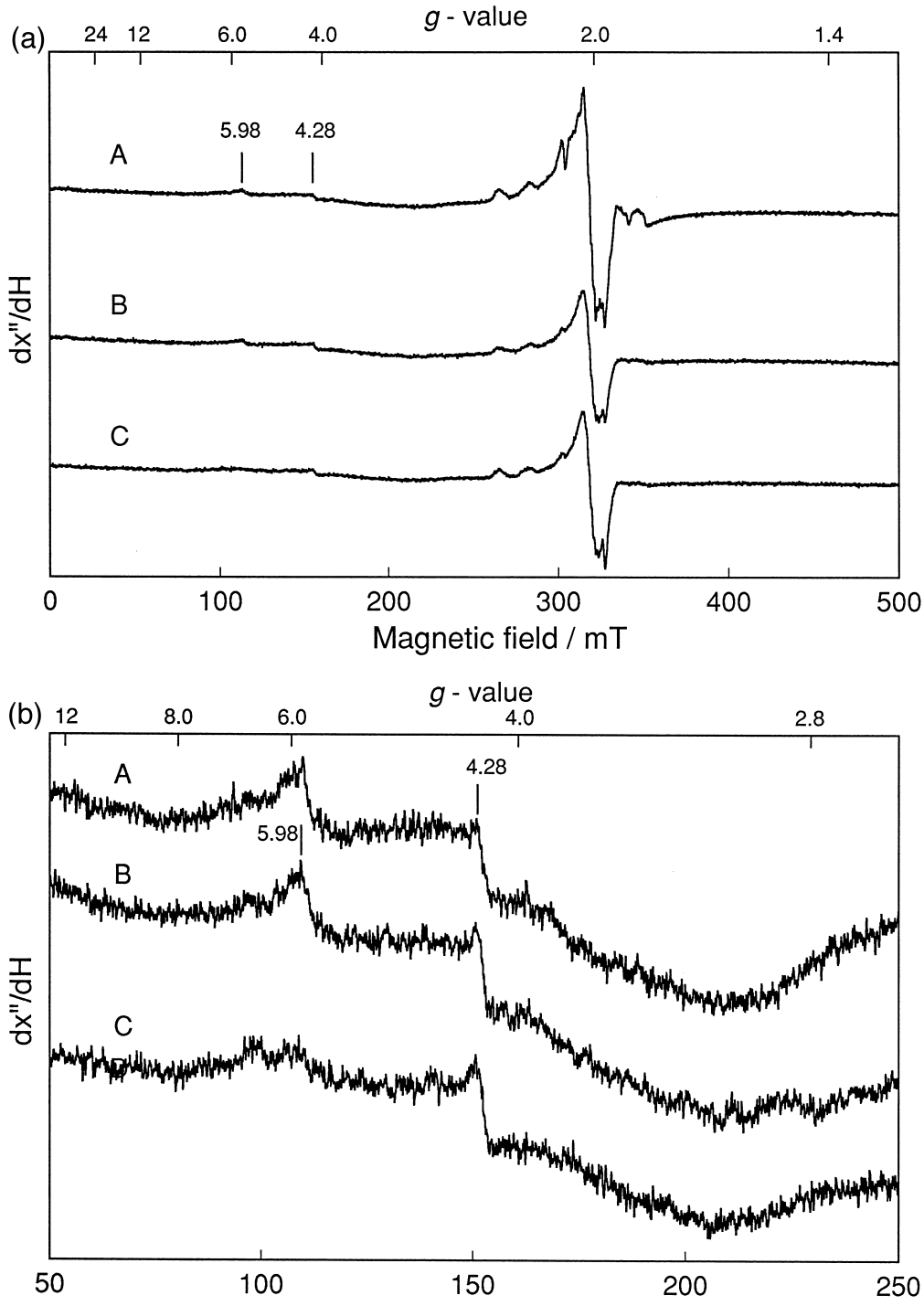
was observed in the precipitate. From the membrane fractions of the cells, pMMO was solubilized with various alkyl glycoside detergents. As shown in Table 1, ca. 2–5% of pMMO was solubilized when *n*-dodecyl-*β*-D-maltoside, sucrose monocaprate or sucrose monolaurate was used. In this study, *n*-dodecyl-*β*-D-maltoside was used to solubilize the enzyme from membranes. To optimize solubilization of pMMO from membranes, the effects of detergent concentration, temperature and incubation time of solubilization were examined. pMMO was solubilized only 5.7% of membrane fractions with *n*-dodecyl-*β*-D-maltoside.

Table 2 shows that a detergent-to-protein weight ratio of 0.92 mg detergent/mg protein

Fig. 5. (a) X-band ESR spectra of isolated pMMO from *M. trichosporium* OB3b during the course of anaerobic redox titration with duroquinol and after exposure to dioxygen and acetylene (at a broad magnetic field). The protein concentration was 2.1 mg protein/ml in 25 mM MOPS (pH 7.0) buffer containing 0.01% *n*-dodecyl-*β*-D-maltoside. A: before addition of duroquinol under anaerobiccondition. B: after addition of duroquinol. C: after exposing the sample (B) to dioxygen and acetylene for 10 min at 30°C. ESR spectrum was recorded at 6.44 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 8.993 GHz. (b) X-band ESR spectra of isolated pMMO from *M. trichosporium* OB3b during the course of anaerobic redox titration with duroquinol and after exposure to dioxygen and acetylene (at a low magnetic field). The protein concentration was 2.1 mg protein/ml in 25 mM MOPS (pH 7.0) buffer containing 0.01% *n*-dodecyl-*β*-D-maltoside. A: before addition of duroquinol under anaerobic condition. B: after addition of duroquinol. C: after exposing the sample (B) to dioxygen and acetylene for 10 min at 30°C. ESR spectrum was recorded at 6.44 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 8.993 GHz.

was optimal to solubilize active pMMO. At high concentration of *n*-dodecyl-*β*-D-maltoside, pMMO activity decreased remarkably.

The effect of temperature for the solubilization of pMMO from membranes indicated the optimum temperature to be 4°C (data not



shown). Increasing the temperature decreased pMMO activity, possibly due to instability of the enzyme pMMO. The solubilization duration did not affect the yields of solubilization, they remained unchanged. In the study, the membrane fractions was solubilized with 0.92 mg detergent/mg protein for 45 min at 4°C under anaerobic conditions.

Membrane protein containing pMMO solubilized with *n*-dodecyl-β-D-maltoside was loaded on to a DEAE Sepharose Fast Flow column (10 × 110 mm) equilibrated with 25 mM MOPS (pH 7.0). pMMO did not bind to the column and eluted in the flowthrough fractions. The flowthrough fractions was loaded on to a Phenyl Sepharose Fast Flow column (10 × 90 mm) and the pMMO activity was found to bind to the column. Then, the enzyme was eluted with a linear gradient of *n*-dodecyl-β-D-maltoside concentration from 0.01 to 0.9%. The results are summarized in Table 3. The pMMO obtained has a specific activity of 0.469 nmol/min/mg protein, and has been purified 2.15-fold.

### 3.2. Estimation of molecular weight of pMMO

The molecular weight of pMMO was measured by using gel filtration and SDS–PAGE methods. From the results shown in Fig. 1, the molecular weight of the enzyme was estimated to be approximately 326 kDa.

SDS–PAGE of the isolated enzyme showed that pMMO contained two bands of molecular masses of 25 and 41 kDa, as well as trace contaminating polypeptide with molecular mass of 26 kDa (Fig. 2). These polypeptides were known as the subunits of pMMO from *M. trichosporium* OB3b, and are similar to the molecular masses of two subunits 27 and 47 kDa in pMMO from *M. capsulatus* (Bath) [7,9].

### 3.3. Content of metal ions in pMMO

The content of metal ions such as copper and iron were measured by using ICP–AES analysis. The isolated pMMO from *M. trichosporium* OB3b contained 0.9 iron atom and 12.8 copper atoms/molecule. The ratio of the metals contained in the enzyme is as follows—copper:iron = 13:1.

### 3.4. ESR spectrum of pMMO from M. trichosporium OB3b

Fig. 3 shows the ESR spectra of isolated pMMO from *M. trichosporium* OB3b. The ESR spectra contained a weak high-spin iron signal ($g = 5.98$) and a type II copper signal ($g_{\|} = 2.24$, $A_{\|} = 18.4$ mT, $g_{\perp} = 2.06$) observed in isolated pMMO. Nguyen et al. reported that anaerobic titration of highly oxidized membranes from *M. capsulatus* (Bath) by dithionite leads to a decrease in the intensity of the copper ESR signal according to partially reduced copper cluster species, and an appearance of a multiple hyperfine structure at $g = 2.06$ [10]. They suggested that this hyperfine structure indicated the splitting arise from the coupling of an unpaired electron spin to three equivalent $I = 3/2$ nuclear spins, i.e., it may have origin in a trinuclear copper cluster. As shown in Fig. 4, the multiple hyperfine structure ($|A| = 1.45$ mT) at $g = 2.06$ existed in the ESR spectrum of pMMO from *M. trichosporium* OB3b. In this spectra, the 10 nuclear hyperfine lines exist, and the spectral intensity distribution of these hyperfine lines was an approximate ratio of 1:3:6:10:12:12:10:6:3:1. These results support that the hypothesis of Nguyen et al. [11] that the hyperfine splitting pattern has origin in a two-electron reduced trinuclear copper cluster. Thus, pMMO from *M. trichosporium* OB3b contains a trinuclear copper cluster.

As shown in Fig. 5a, anaerobic and successive addition of excess amounts of duroquinol resulted in a decrease in the intensity of the copper ESR signal (Fig. 5a-B). Thus, the copper cluster of pMMO reduced by duroquinol as a reductant of pMMO. This result suggests that the copper cluster in pMMO plays as an electron transfer or active site of pMMO. Treatment of isolated pMMO with acetylene, dioxygen and

duroquinol results in irreversible inhibition of pMMO (data not shown). Upon exposure of this reduced sample to dioxygen and acetylene at 30°C, the copper ESR signal increased (Fig. 5a-C), indicating that the copper cluster oxidized by excess dioxygen. As shown in Fig. 5b, the ESR intensity with a high-spin iron signal ($g = 5.98$) did not change by successive addition of excess amounts of duroquinol. However, upon exposure of this reduced sample to dioxygen and acetylene at 30°C, the high-spin iron signal disappeared ($g = 5.98$) (Fig. 5b-C). Acetylene inhibits pMMO activity by binding ketene, an intermediate of acetylene oxidation, with the active site of pMMO [10]. 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.

## Acknowledgements

*M. trichosporium* OB3b was kindly provided by Professor J.D. Lipscomb of University of Minnesota. This present work is partly defrayed by the Grant-in-Aid for Scientific Research on Priory-Areas-Research from the Ministry of Education, Science, Sports and Culture of Japan (08231226).

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