Effect of methanobactin on the activity and electron paramagnetic resonance spectra of the membrane-associated methane monooxygenase in *Methylococcus capsulatus* Bath

Dong W. Choi,¹ William E. Antholine,² Young S. Do,¹ Jeremy D. Semrau,³ Clint J. Kisting,¹ Ryan C. Kunz,^{1†} Damon Campbell,⁴ Vinay Rao,⁴ Scott C. Hartsel⁴ and Alan A. DiSpirito¹

Correspondence
Alan A. DiSpirito
aland@iastate.edu

¹Department of Biochemistry, Biophysics and Molecular Biology, 4164 Molecular Biology Building, Iowa State University, Ames, IA 50011-3211, USA

²Department of Biophysics, Medical College of Wisconsin, Milwaukee, WI 53226, USA

³Department of Civil and Environmental Engineering, University of Michigan, Ann Arbor, MI 48109-2125, USA

⁴Department of Chemistry, University of Wisconsin-Eau Claire, Eau Claire, WI 54702, USA

Improvements in the purification of methanobactin (mb) from either *Methylosinus trichosporium* OB3b^T or *Methylococcus capsulatus* Bath resulted in preparations that stimulated methane-oxidation activity in both whole-cell and cell-free fractions of *Methylococcus capsulatus* Bath expressing the membrane-associated methane monooxygenase (pMMO). By using washed membrane fractions with pMMO activities in the 290 nmol propylene oxidized min⁻¹ (mg protein)⁻¹ range, activities approaching 400 nmol propylene oxidized min⁻¹ (mg protein)⁻¹ were commonly observed following addition of copper-containing mb (Cu-mb), which represented 50–75% of the total whole-cell activity. The stimulation of methane-oxidation activity by Cu-mb was similar to or greater than that observed with equimolar concentrations of Cu(II), without the inhibitory effects observed with high copper concentrations. Stimulation of pMMO activity was not observed with copper-free mb, nor was it observed when the copper-to-mb ratio was <0.5 Cu atoms per mb. The electron paramagnetic resonance (EPR) spectra of mb differed depending on the copper-to-mb ratio. At copper-to-mb ratios of <0.4 Cu(II) per mb, Cu(II) addition to mb showed an initial coordination by both sulfur and nitrogen, followed by reduction to Cu(I) in <2 min. At Cu(II)-to-mb ratios between 0.4 and 0.9 Cu(II) per mb, the intensity of the Cu(II) signal in EPR spectra was more representative of the Cu(II) added and indicated more nitrogen coordination. The EPR spectral properties of mb and pMMO were also examined in the washed membrane fraction following the addition of Cu(II), mb and Cu-mb in the presence or absence of reductants (NADH or duroquinol) and substrates (CH₄ and/or O₂). The results indicated that Cu-mb increased electron flow to the pMMO, increased the free radical formed following the addition of O₂ and decreased the residual free radical following the addition of O₂ plus CH₄. The increase in pMMO activity and EPR spectral changes to the pMMO following Cu-mb addition represent the first positive evidence of interactions between the pMMO and Cu-mb.

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[†]Present address: Department of Biochemistry, Beadle Center, University of Nebraska, Lincoln, NE 68588-0664, USA.

Abbreviations: Cu-mb, copper-containing methanobactin; EPR, electron paramagnetic resonance; mb, methanobactin; MALDI-TOF, matrix-assisted laser desorption ionization–time-of-flight; MMO, methane monooxygenase; pMMO, membrane-associated methane monooxygenase; sMMO, soluble methane monooxygenase.

Supplementary material is available with the online version of this paper.

INTRODUCTION

Methanobactin (mb), previously called copper-binding compound or copper-binding peptide, was initially identified in the methanotroph *Methylococcus capsulatus* Bath in association with the membrane-associated or particulate methane monooxygenase (pMMO) (Zahn & DiSpirito, 1996). This copper-binding molecule was later identified in

the extracellular fractions of both *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b^T (D. W. Choi, Y. S. Young, J. D. Semrau, W. E. Antholine, C. J. Kisting, S. C. Hartsel & A. A. DiSpirito, unpublished results; DiSpirito *et al.*, 1998; Fitch *et al.*, 1993; Kim *et al.*, 2004, 2005; Téllez *et al.*, 1998). Copper-containing mb (Cu-mb) was originally proposed as a cofactor of the pMMO, based on the irreversible loss of pMMO activity following separation (Choi *et al.*, 2003; Zahn & DiSpirito, 1996). In this model, the core enzyme was composed of three polypeptides with approximate molecular masses of 45 000 (α subunit), 25 000 (β subunit) and 20 000 (γ subunit) Da in a 1 : 1 : 1 ratio. The $\alpha\beta\gamma$ subunits of the pMMO were believed to contain two type II Cu(II) and two electron paramagnetic resonance (EPR)-silent Fe atoms. Active preparations also contained 8–13 Cu-mb. The crystal structure of the core enzyme has recently been determined (Lieberman & Rosenzweig, 2005; Sommerhalter *et al.*, 2005). The crystal structure showed that the enzyme is a trimer, ($\alpha\beta\gamma$)₃. Each $\alpha\beta\gamma$ monomer contained one type II Cu(II) atom and two copper atoms that appear to be spin-coupled, similar to the Cu_A in cytochrome *c* oxidase, or a modified type III site associated with the β subunit (Boas, 1984; Lieberman & Rosenzweig, 2005). The enzyme was also reported to contain one Zn(II) atom coordinated between the α and β subunits (Lieberman & Rosenzweig, 2005). The source of the Zn(II) was proposed to be the crystallization buffer, as no Zn(II) was associated with the enzyme preparations before crystallization. Further, the results of these studies suggested that the Zn(II) may have replaced another metal, such as Cu or Fe. The pMMO preparation used in the crystallization study was inactive, so the absence of Cu-mb in the crystal structure was not surprising, considering that Cu-mb is easily dissociated from the $\alpha\beta\gamma$ polypeptides of the pMMO (Choi *et al.*, 2003; Zahn & DiSpirito, 1996).

Previous studies from this laboratory have shown that high-activity pMMO preparations require co-purification of Cu-mb with the three pMMO polypeptides (Choi *et al.*, 2003). Additional evidence for a potential involvement of Cu-mb in methane oxidation comes from the culture conditions used to stabilize cell-free pMMO activity, which result in an increased concentration of membrane-associated Cu-mb (Choi *et al.*, 2003; Zahn & DiSpirito, 1996). Studies on the role of Cu-mb in methane oxidation by the pMMO have been limited, as the only direct correlation between these two proteins was the irreversible loss of methane-oxidation activity following dissociation. Cu-mb has been shown to have superoxide dismutase activity, which may account for its stabilizing effects on cell-free pMMO activity (Choi *et al.*, 2003). Recent improvements in the stabilization of the pMMO in cell-free fractions (Basu *et al.*, 2003; Choi *et al.*, 2003; D. W. Choi, Y. S. Young, J. D. Semrau, W. E. Antholine, C. J. Kisting, S. C. Hartsel & A. A. DiSpirito, unpublished results), as well as in the isolation of mb (this report) and Cu-mb (Kim *et al.*, 2005), however, have caused us to reconsider the potential role of mb and Cu-mb in methane oxidation. In this study, we show that

Cu-mb stimulates pMMO activity, and the results suggest that the stimulation is due to an increased rate of electron flow to the type II Cu(II) centre(s) of the pMMO.

METHODS

Organisms, culture conditions and isolation of membrane fractions. *Methylosinus trichosporium* OB3b^T and *Methylococcus capsulatus* Bath were cultured for mb isolation in nitrate minimal salts (NMS) medium containing 0, 0.2 or 1 μ M added CuSO₄, as described previously (Choi *et al.*, 2003). The initial copper concentration in NMS medium with no added CuSO₄ was 0.29 ± 0.04 μ M. The cultures were grown in batch mode to an OD₆₀₀ between 0.7 and 1.2 prior to harvesting for mb. When the OD₆₀₀ reached the desired level, 80 % of the fermenter was harvested and replaced with fresh NMS medium. *Methylococcus capsulatus* Bath was also cultured in NMS medium that contained a final CuSO₄ concentration of either 60 or 80 μ M, as described previously (Choi *et al.*, 2003). Washed membranes from *Methylococcus capsulatus* Bath were isolated under anaerobic conditions as described by Choi *et al.* (2003).

Isolation of mb. Cu-mb and mb were prepared from the spent medium of *Methylosinus trichosporium* OB3b^T or *Methylococcus capsulatus* Bath. For each harvest, the spent medium was centrifuged twice at 9000 g for 20 min to remove residual cells. At this stage, the spent medium was either loaded onto a 7 × 20 cm Dianion HP-20 column (Supelco) or stabilized by the addition of copper as described by Kim *et al.* (2004), except that the final concentration of added copper was reduced from 10 to 1 mM. The Dianion HP-20 column was washed with two column volumes of H₂O, eluted with 60 % methanol:40 % water (v/v) and lyophilized. At this stage of purification, mb represented >97 % of the material absorbing at 214 or 280 nm and no other chromophores with absorption maxima above 280 nm were present. Purity of mb samples was checked at this stage by HPLC, matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry and UV-visible absorption spectra. Unless indicated, the freeze-dried samples following chromatography on Dianion HP-20 columns were the source of mb or Cu-mb used in this study. Selected samples were purified further by reverse-phase HPLC on a Beckman Gold HPLC system by using a SupelcoSil LC-18 (25 cm × 4.6 mm, 5 μ m) column at a flow rate of 1.0 ml min⁻¹, with 10 mM sodium phosphate buffer, pH 6.6 (solvent A) and 80 % acetonitrile/H₂O (solvent B) as the mobile phase. A linear gradient consisting of an initial solvent B concentration of 5 % following injection to 35 % solvent B at 50 min and 100 % at 55 min was used in this purification step.

Sample purity and metal content of final samples were based on the UV-visible absorption spectra, on metal analysis and on molecular masses as determined by MALDI-TOF MS, of the fractions before and after separation by reverse-phase HPLC.

Molecular-mass determinations. Solution molecular mass of mb samples was determined on a Superdex Peptide HR 10/30 column (Pharmacia/LKB) equilibrated with MilliQ water (Millipore), pH 6.8. The column was calibrated by using blue dextran, orange G, bradykinin (1240 Da), rennin substrate (1759 Da), insulin (5734 Da) and horse-heart cytochrome *c* (12 500 Da).

MALDI-TOF mass spectra were obtained on a Voyager-DE PRO Biospectrometry Workstation 6075 (PerSeptive Biosystems, Inc.). Analyses were performed in the reflector-positive mode with time-delayed extraction (200 ns). Acquisition-mass range was typically between 500 and 5000 Da, with laser intensities between 1900 and 2100 intensity units. The matrix solution used was *p*-nitroaniline (Fluka Chemika) (35 mM in a 1:1 mixture of water:ethanol at pH 6.5). Typically, 1 μ l of an mb solution (5 mg ml⁻¹) was diluted 1:10 with

p-nitroaniline matrix. A 2 µl aliquot of the analyte/matrix solution was spotted onto a stainless-steel sample plate and allowed to dry before analysis.

Enzyme activity, isolation of cell fraction and protein determinations. Methane monooxygenase (MMO) activity was determined by the epoxidation of propylene, as described previously (Choi *et al.*, 2003), and measured either in the liquid phase on an SRI 8610C GC system (SRI Instruments) equipped with a flame-ionization detector and an 8' × 0.085'' HaySep D column, or in the gas phase on a Varian 3900 (Varian Corporation) equipped with a flame-ionization detector and a 30 m × 0.53 mm Supel-Q plot column. Isolation of the cell-free fraction, copper determinations and protein determinations were carried out as described previously (Choi *et al.*, 2003). In addition to propylene-oxidation activity in the soluble fraction, soluble MMO (sMMO) activity was monitored by the formation of naphthol from naphthalene as described by Brusseau *et al.* (1990).

The effects of mb on pMMO activity were examined in the washed membrane fraction from *Methylococcus capsulatus* Bath by using mb from *Methylosinus trichosporium* OB3b^T. Washed membrane fractions from *Methylococcus capsulatus* Bath were used, as procedures for the isolation of membrane fractions with high pMMO activity have only been developed in this species (Basu *et al.*, 2003; Choi *et al.*, 2003; Yu *et al.*, 2003). mb from *Methylosinus trichosporium* OB3b^T was used in these studies for a variety of reasons. First, the EPR spectra of Cu-mb from both methanotrophs were identical (W. E. Antholine, D. W. Choi, Y. S. Young & A. A. DiSpirito, unpublished results). Second, stimulation of pMMO activity by Cu-mb was 10–20 % higher using Cu-mb from *Methylosinus trichosporium* OB3b^T than that observed with equimolar concentrations of Cu-mb from *Methylococcus capsulatus* Bath (results not shown). Third, the yields of mb from the spent medium of *Methylosinus trichosporium* OB3b^T were generally several-fold higher than observed with *Methylococcus capsulatus* Bath (Choi *et al.*, 2003; DiSpirito *et al.*, 1998; Kim *et al.*, 2005; Zahn & DiSpirito, 1996). Fourth, the mb from *Methylosinus trichosporium* OB3b^T is the best-characterized mb, structurally (Kim *et al.*, 2004, 2005), spectrally (D. W. Choi, Y. S. Young, J. D. Semrau, W. E. Antholine, C. J. Kisting, S. C. Hartsel & A. A. DiSpirito, unpublished results; DiSpirito *et al.*, 1998; Kim *et al.*, 2005) and thermodynamically (D. W. Choi, Y. S. Young, J. D. Semrau, W. E. Antholine, C. J. Kisting, S. C. Hartsel & A. A. DiSpirito, unpublished results). Lastly, the Cu-mb from *Methylosinus trichosporium* OB3b^T is more soluble than Cu-mb samples from *Methylococcus capsulatus* Bath, which tend to precipitate in solutions at concentrations above 10 µM.

Cu-mb and substrate effects on the EPR spectra of washed membranes. Membrane samples isolated under anaerobic conditions from *Methylococcus capsulatus* Bath were prepared for EPR studies in 6 ml amber serum vials in an anaerobic chamber (Coy Laboratory), using 5 % hydrogen with 95 % argon. In samples containing reductants (either NADH or duroquinol), 2.12 nmol reductant was added (mg membrane protein)⁻¹. The concentration of reductant added was based on an estimated pMMO content of 20 % in the washed membrane samples. For samples containing added Cu(II) or Cu-mb, the optimal molar ratio of Cu(II) or Cu-mb to pMMO was chosen based on the concentration yielding the highest propylene-oxidation activity. The samples were mixed and the hypovials were sealed with Teflon-coated silicon septa. Substrates (2 ml O₂, 2 ml CH₄ or 2 ml of each) were added with gas-tight syringes and the samples were incubated for 5 min at room temperature with shaking. After incubation, samples were transferred to EPR tubes, sealed and taken out of the anaerobic chamber. Samples in EPR tubes were then frozen in liquid nitrogen and stored on dry ice.

UV-visible absorption spectroscopy. UV-visible absorption spectroscopy was performed as described previously (Choi *et al.*,

2003). Kinetic photodiode array spectral series were taken by using a microvolume stopped-flow reaction analyser (Applied Photophysics and SX.18MV). Spectral series were measured at 2.0 °C from 275 to 500 nm by using a diode array detector with an integration time of 2.56 ms. The mixing chamber had a 1.0 cm path length and the monochromator slit width was fixed at 1.0 mm entry and 1.0 mm exit. All samples were protected from ambient light to prevent possible photo-oxidation. Pro-K SVD and global analysis software from Applied Photophysics was used for data analysis (Henry & Hofrichter, 1992).

EPR spectroscopy. Q-, S- and X-band EPR spectra were obtained as described by Yuan *et al.* (1999).

RESULTS

Isolation of mb in the absence of copper

The isolation of Cu-mb has recently been reported by Kim *et al.* (2005). This purification procedure solved a number of problems associated with earlier purification procedures, such as samples of varying purity and breakdown products (DiSpirito *et al.*, 1998; Kim *et al.*, 2005; Zahn & DiSpirito, 1996). In addition, in contrast to earlier studies, the Cu-mb isolated by the procedures described in Methods or by Kim *et al.* (2005) stimulated pMMO activity in whole-cell and washed membrane fractions (see below). However, the mb samples isolated by the procedure of Kim *et al.* (2004, 2005) are copper-saturated (i.e. 1.2 ± 0.16 Cu per mb) and dialysis against Na₂EDTA only removed approximately 90 % of the Cu associated with mb (results not shown). This dialysis treatment also altered the thermodynamic and spectral properties of the sample (D. W. Choi, Y. S. Young, J. D. Semrau, W. E. Antholine, C. J. Kisting, S. C. Hartsel & A. A. DiSpirito, unpublished results). The purification procedure was simplified to obtain low-copper-containing mb samples and to avoid the breakdown products observed in a previous purification procedures. A one-column procedure was used to purify mb from the spent medium (described in Methods). mb samples purified by this procedure represented >97 % of the material absorbing at 214 or 280 nm and contained no other chromophores (Choi *et al.*, 2005a, b). The purified mb samples from this procedure contained 0.01–0.02 Cu per mb and were stable if the samples were freeze-dried and stored in the dark at –20 °C. Aqueous solutions of mb were also stable for one freeze-thaw cycle if frozen in liquid nitrogen and stored in the dark at –80 °C. Samples were stored in the dark or in amber bottles, as exposure to light resulted in photoinactivation of the sample (data not shown).

Effects of Cu-mb on pMMO activity

The effects of Cu(II), mb, Cu-mb and mb plus Cu(II) at different molar ratios on pMMO activity in *Methylococcus capsulatus* Bath are shown in Fig. 1. The cells and membrane fractions used in this component of the study were from *Methylococcus capsulatus* Bath cultured in medium with a final copper concentration of either 60 µM CuSO₄, where the expression levels of pMMO are highest, or 80 µM

Fig. 1. Effect of Cu-mb on the rate of propylene oxidation by *Methylococcus capsulatus* Bath expressing the pMMO. (a) Effect of mb (□), Cu-mb (●) and CuSO₄ (△) on whole-cell propylene-oxidation rates in *Methylococcus capsulatus* Bath. Cells were cultured in a chemostat to an OD₆₀₀ of 1.2 as described previously (Choi *et al.*, 2003) and harvested when the copper concentration added to the medium reached 80 μM. Protein concentration in reaction vials was 1.4 mg ml⁻¹. (b) Effect of CuSO₄ (△) and Cu-mb (●) on propylene-oxidation rates in the washed membrane fraction of cells grown and harvested as described in (a). (c) Effect of Cu-mb (●) and Cu(mb)₂ (■) on propylene-oxidation rates in the washed membrane fraction of cells grown and harvested as described in (a). (d) Effect of Cu(II)-to-mb molar ratio on the rate of methane oxidation in the washed membrane fraction from cells cultured in 60 (○) or 80 (●) μM Cu(II). The lines labelled 'No mb (80 μM)' and 'No mb (60 μM)' illustrate the activity in the washed membrane fraction with no added mb.

CuSO₄, which has been shown to saturate the cells with copper (Choi *et al.*, 2003). The copper and mb concentrations in membrane samples of cells cultured under these conditions were approximately 250 nmol Cu (mg protein)⁻¹ (Choi *et al.*, 2003) and 150 nmol mb (mg protein)⁻¹. Even in cells cultured under these high-copper conditions, the addition of Cu(II) usually stimulated pMMO activity, although higher concentrations of Cu(II) were always inhibitory (Fig. 1a). In whole-cell samples, the stimulation of pMMO by copper was three- to fourfold higher if added as Cu-mb (Fig. 1a). An optimal Cu-mb-to-cell ratio was always observed, followed by a small decrease in pMMO activity as the Cu-mb-to-cell ratio was increased further. However, even at high Cu-mb concentrations, the addition of Cu-mb stimulated pMMO activity in whole-cell samples and never showed the inhibition observed with Cu(II). This stimulation was only observed with Cu-mb; mb itself was slightly inhibitory to whole-cell propylene-oxidation rates (Fig. 1a).

The washed membrane fractions from cells cultured above 60 μM CuSO₄ showed initial propylene-oxidation activities

in the range of 75–230 nmol min⁻¹ (mg protein)⁻¹, before Cu(II) or Cu-mb additions. These propylene-oxidation rates are several-fold higher than previously reported rates (Basu *et al.*, 2003; Chan *et al.*, 2004; Choi *et al.*, 2003; Lieberman *et al.*, 2003) and are dependent on copper-addition rates during growth. Fig. 1(b and c) represent two different membrane preparations showing high- and low-activity membrane samples. Maintaining anaerobic conditions, eliminating light and maintaining temperatures below 4 °C during cell lysis aided in the stabilization of pMMO activity. Stimulation of pMMO activity in the washed membrane fraction by Cu(II) or by Cu-mb was similar to that observed in whole-cell samples, except that the stimulatory and inhibitory effects were more pronounced (Fig. 1b and c). The stimulation of propylene oxidation by Cu-mb varied from 35 to 140 %, depending on the membrane preparation, with activities approaching 400 nmol min⁻¹ (mg protein)⁻¹ in some membrane preparations following Cu-mb addition. In general, the stimulation of pMMO activity by Cu-mb in washed membrane fractions increased proportionally with the copper concentrations used during growth. In addition, the percentage stimulation of pMMO

activity by Cu-mb increased with the initial activity of the membrane preparation.

Several recent experiments have suggested that mb may initially bind copper as a homodimer, i.e. as $\text{Cu}(\text{mb})_2$, followed by the binding of a second $\text{Cu}(\text{II})$, resulting in a final molar ratio of 1 copper atom per mb, i.e. Cu-mb (D. W. Choi, Y. S. Young, J. D. Semrau, W. E. Antholine, C. J. Kisting, S. C. Hartsel & A. A. DiSpirito, unpublished results). To examine whether the pMMO in the washed membrane fraction responded differently to $\text{Cu}(\text{mb})_2$, the effect of $\text{Cu}(\text{mb})_2$ on pMMO activity was also examined (Fig. 1c). In contrast to Cu-mb, which stimulated pMMO activity, $\text{Cu}(\text{mb})_2$ was inhibitory to pMMO activity (Fig. 1c). To further examine this property, the effect of mb with different copper-to-mb molar ratios on pMMO activity in the washed membrane samples was examined (Fig. 1d). In general, mb was inhibitory to pMMO activity at copper-to-mb molar ratios of <0.6 copper atoms per mb and stimulatory at concentrations above $0.6\text{--}0.8$ copper atoms per mb (Fig. 1d).

EPR spectra of mb

In an attempt to identify Cu-mb spectrally in the washed membrane fraction of methanotrophs, the EPR spectral properties of purified mb were examined by X-, Q- and S-band EPR. The EPR spectrum of Cu-mb at the conventional microwave frequency (X-band) and at a higher (Q-band) and a lower (S-band) frequency confirms the binding of cupric ion (Fig. 2). Two observations distinguish the X-band spectrum of Cu-mb. First, the lines in the low-field region [$g_{\parallel}=2.23$, $A_{\parallel}=185\text{ G}$ ($1\text{ G}=10^{-4}\text{ T}$)] were broader than usual (Boas, 1984). This indicates more strain in the axial direction than is observed from most type II cupric complexes. These lines in the $g_{\parallel}$ region were sharper at a lower microwave frequency (S-band trace in Fig. 2) and broader at higher microwave frequencies (Q-band). For Q-band analysis, the $g_{\parallel}$ lines were broad and not detected (not shown). In the X-band spectrum, there were lines at high field, split by 16 G . These lines split by 16 G were also evident in the S-band spectrum on the S-shaped signal from the $g_{\perp}$ region. The first harmonic of the S-band trace emphasizes the sharp lines. The Q-band spectrum also has sharp lines on the high-field side, which are attributed to the $g_{\perp}$ region. Superimposed on the Cu-mb lines were five or six Mn lines and a free-radical signal that were not detected at X- or S-band. Q-band spectra contained Mn and free-radical signals that were considered background signals. The first harmonic of the Q-band spectrum emphasizes the sharp lines. They are part of the $g_{\perp}$ region from an axial-symmetric site and not from g_x for a rhombic site with three g values (g_z , g_y , g_x), because the g_x peak would be superimposed about the free radical in the Q-band spectrum if this was a true g value. Presumably, the shoulder on the high-field side in the X-band spectrum was an overshoot line that disappears in the Q-band spectrum, as expected for an overshoot line at X-band. As there were at least 10 lines split by 16 G that were resolved and probably more unresolved

Fig. 2. Q-, S- and X-band EPR spectra of Cu-mb at 77 K. The regions with lines split by 16 G are circled. $A_{\parallel}$ and $g_{\parallel}$ are indicated by lines and arrows. Spectrometer conditions were as follows. X-band: microwave frequency, 9.195 GHz ; microwave power, 5 mW ; temperature, 77 K ; four scans averaged. S-band: microwave frequency, 3.334 GHz ; microwave power, 16 dB (incident power, 10 mW); temperature, -140°C ; four scans averaged. Q-band: microwave frequency, 34.786 GHz ; microwave power, 26 dB ; temperature, -140°C ; nine scans averaged. The modulation amplitude was 5 G , scan time was 4 min and the time constant was 0.128 s for all spectra.

lines in the spectra, these lines were attributed to super-hyperfine lines due to nitrogen-donor atoms in addition to protons that are close to the cupric ion. It is surmised that the cupric-binding site was formed from three or four nitrogen-donor atoms, due to the number of lines resolved, the $g_{\parallel}$ value of 2.23 and the $A_{\parallel}$ value of 185 G .

Consistent with earlier studies (DiSpirito *et al.*, 1998; Zahn & DiSpirito, 1996) and recent X-ray photoelectron spectroscopy (Kim *et al.*, 2004), the EPR spectra of mb showed that the copper associated with mb was predominantly Cu(I) and not EPR-detectable (Figs 3 and 4). In copper-titration experiments, the mb concentration was 4 mM and CuSO_4 additions started at $240\text{ }\mu\text{M}$ (0.06 molar equivalents), which should have provided a strong signal if the copper remained as Cu(II). However, in the spectra following the addition of 0.06 , 0.125 , 0.25 and 0.3 molar equivalents of Cu(II), the signal remained lower than expected and the residual Cu(II) has $g_{\parallel}$ about 2.20 and $A_{\parallel}$ about 180 G , indicative of thiol coordination, but there appears to be more than a single cupric site. The spectra in these samples were complex and fell between predictable values for the projected $2\text{N}2\text{S}$ and $3\text{N}1\text{S}$ coordination, which may be due to the oxidation of one of the sulfurs associated with the imidazole group. At Cu(II) additions above 0.4 molar equivalents, the intensity of the spectra was more in keeping with the concentration of

Fig. 3. X-band EPR spectra at 77 K of mb (4 mM) following the addition of 0.06, 0.125, 0.25, 0.33, 0.4 and 0.5 molar equivalents of CuSO_4 . A_{II} and g_{II} are indicated by lines and arrows. Experimental conditions: modulation amplitude, 5 G; modulation frequency, 100 kHz; microwave power, 5 mW; temperature, 77 K. Note: $\times$ refers to the amount of signal amplification for presentation purposes.

added copper and the spectra have values of $g_{\text{II}}=2.23$ and $A_{\text{II}}=185$ G, indicating more nitrogen coordination.

Time-course experiments showed that all the Cu(II) added was reduced to Cu(I) in <10 min (Fig. 4). The Cu(I) associated with mb was stable for hours in oxygenated solutions and required molar excess of H_2O_2 to oxidize the Cu(I) to Cu(II) (Fig. 4). The Cu(II) remained associated with mb and can be observed by UV-visible absorption spectroscopy (W. E. Antholine, D. W. Choi, Y. S. Young & A. A. DiSpirito, unpublished results). Upon extended incubation with H_2O_2 , an EPR signal for the cupric site was obtained with $g_{\text{II}}=2.23$ and $A_{\text{II}}=185$ G, but without the resolved superhyperfine structure, possibly because there was more strain, multiple cupric sites or aggregation. In order to prevent free-radical reactions close to the cupric site, HCO_3^- was added before H_2O_2 was added. Oxygen radicals react with HCO_3^- to form CO_2 and diffuse away from the cupric ion, preventing any reactivity with copper. The spectrum obtained after 30 min was better-defined in the g_{II} region with $g_{\text{II}}=2.20$ and $A_{\text{II}}=182$ G, along with two or three nitrogen-donor atoms.

Cu(II), Cu-mb and substrate effects on the EPR spectra in membrane samples

The stimulatory effect of Cu-mb on propylene oxidation by the pMMO suggests a potential role of Cu-mb in methane oxidation by the pMMO. To examine the possible cause for this stimulation, the EPR spectral properties in the washed membrane fraction were examined following the addition of Cu-mb in the presence and absence of a reductant, NADH, and substrates, CH_4 and/or O_2 . To minimize

Fig. 4. X-band EPR spectra of 2.7 mM mb following the addition of 0.7 mM CuSO_4 . Following copper addition, samples were incubated for 1, 10, 56 and 266 min at room temperature, then frozen in liquid nitrogen. In samples (2.7 mM mb plus 0.7 mM CuSO_4) treated with H_2O_2 , the H_2O_2 was added to a final concentration of 2.7 or 10 mM and incubated for 4, 10, 20 or 30 min at room temperature, then frozen in liquid nitrogen. A_{II} and g_{II} are indicated by lines and arrows. Experimental conditions: modulation amplitude, 5 G; modulation frequency, 100 kHz; microwave power, 5 mW; temperature, 77 K.

variability in the spectral characterization of the pMMO, the membranes were isolated and reaction vials were prepared under anaerobic conditions. In reaction mixtures containing O_2 and/or CH_4 , the gases were added with gas-tight syringes to closed 6 ml serum vials in the anaerobic chamber. Under these conditions, the EPR-detectable Cu(II) in the pMMO was reduced by 50–65% and the addition of NADH did not result in additional reduction of the type II Cu(II) centres associated with the pMMO [Fig. 5a, traces (i) and (ii)]. The inability to reduce the remaining copper centres may represent the physiological resting state of the enzyme or it may represent the population of inactive enzyme in these preparations. Addition of CH_4 did not change the spectral properties [Fig. 5a, trace (iii)]. However, in the presence of O_2 , the intensity of the type II Cu(II) signal was increased by approximately 50% and a free-radical signal was generated at $g=2.005$ [Fig. 5a, trace (iv)]. The addition of methane and oxygen resulted in spectra that were similar to the spectra of O_2 alone, but the free-radical signal was reduced by 40% [Fig. 5a, trace (v)]. These results suggest that, as in the sMMO (Wallar & Lipscomb, 1996, 2001), the pMMO activates oxygen before reacting with methane.

Depending on the sample preparation, two different EPR spectral changes were observed following the addition of Cu-mb and NADH. Fig. 5(b) illustrates one of the EPR

Fig. 5. Effect of Cu(II), Cu-mb, NADH, O₂ and CH₄ addition on X-band EPR spectra at 77 K of the cupric site of the washed membrane fraction from *Methylococcus capsulatus* Bath expressing the pMMO isolated under anaerobic conditions. (a) and (b) were from the washed membrane samples used in Fig. 1(b) and samples used in (c) were from washed membrane samples shown in Fig. 1(c). In (a), the gain increased by a factor of 5.76 compared with (b). (a) Effect of substrate additions on EPR spectra of the cupric site of the washed membrane fraction from *Methylococcus capsulatus* Bath: (i) resting; (ii) following the addition of 2.12 nmol NADH (mg membrane protein)⁻¹; (iii) following the addition of 2.12 nmol NADH and 1.9 μmol CH₄ (mg membrane protein)⁻¹; (iv) following the addition of 2.12 nmol NADH and 1.9 μmol O₂ (mg membrane protein)⁻¹; (v) following the addition of 2.12 nmol NADH, 1.9 μmol CH₄ and 1.9 μM O₂ (mg membrane protein)⁻¹. (b) Effect of Cu-mb and substrate additions on EPR spectra of the cupric site of the washed membrane fraction from *Methylococcus capsulatus* Bath: (i) following the addition of 2.12 nmol NADH; (ii) following the addition of 23.5 nmol Cu-mb and 2.12 nmol NADH (mg membrane protein)⁻¹; (iii) following the addition of 23.5 nmol Cu-mb, 2.12 nmol NADH and 1.9 μmol CH₄ (mg membrane protein)⁻¹; (iv) following the addition of 23.5 nmol Cu-mb, 2.12 nmol NADH and 1.9 μmol O₂; (v) following the addition of 23.5 nmol Cu-mb, 2.12 nmol NADH, 1.9 μmol CH₄ and 1.9 μM O₂ (mg membrane protein)⁻¹. (c) Effect of 20 nmol Cu-mb (mg membrane protein)⁻¹ on the lower-activity membrane fractions shown in Fig. 1(c): (i) resting; (ii) following the addition of 2.12 nmol NADH (mg membrane protein)⁻¹; (iii) following the addition of 20 nmol Cu-mb and 2.12 nmol NADH (mg membrane protein)⁻¹; (iv) following the addition of 20 nmol Cu-mb, 2.12 nmol NADH and 1.9 μmol O₂ (mg membrane protein)⁻¹; (v) following the addition of 20 nmol Cu-mb, 2.12 nmol NADH, 1.9 μmol CH₄ and 1.9 μM O₂ (mg membrane protein)⁻¹. Instrument conditions were: modulation frequency, 100 kHz; modulation amplitude, 5 G; time constant, 100 ms; microwave frequency, 9.191 GHz; microwave power, 5.0 mW. Note: × refers to the amount of signal amplification for presentation purposes.

Cu-mb resulted in the near-complete reduction of the type II Cu(II) signal associated with pMMO and the appearance of the free-radical signal at $g = 2.005$ [Fig. 5b, trace (ii)]. The near-complete reduction of the type II Cu(II) signal suggests that the majority of the pMMO in the washed membrane sample was active. The addition of CH₄ did not alter the EPR spectral properties of the membrane in the presence of Cu-mb [Fig. 5b, trace (iii)]. However, the addition of O₂ resulted in the reoxidation of the type II Cu(II) centre(s) of the pMMO and increased the intensity of the free-radical signal at $g = 2.005$ [Fig. 5b, trace (iv)]. Surprisingly, the addition of both O₂ and CH₄ to the membrane fraction in the presence of Cu-mb resulted in the partial oxidation of the type II Cu(II) centre(s) without the formation of the radical signal at $g = 2.005$ [Fig. 5b, trace (v)].

In other membrane preparations, the reduction of the pMMO following addition of Cu-mb was comparatively small (Fig. 5c). Also, as in the previous example (Fig. 5b), the addition of high concentrations of Cu-mb

spectral changes observed in the high-activity membrane preparations. In the presence of a reductant, the addition of

did not increase the intensity of the Cu(II) signal in the washed membrane sample, suggesting that the copper associated with mb remained as Cu(I). In these membrane preparations, the intensity of the radical signal became larger following O₂ addition [Fig. 5c, trace (iv)]. However, in contrast to the membrane series represented in Fig. 5(b), the addition of CH₄ with O₂ only resulted in partial quenching of the radical signal [Fig. 5c, trace (v)]. As a general rule, Cu(II) is known to stimulate pMMO activity in whole-cell as well as in cell-free fractions (Basu *et al.*, 2003; Chan *et al.*, 2004; Choi *et al.*, 2003; Collins *et al.*, 1991; Dalton *et al.*, 1984; Lieberman *et al.*, 2003; Nguyen *et al.*, 1998; Zahn & DiSpirito, 1996). However, in contrast to the copper added as Cu-mb, much of the added Cu(II) appeared as unassociated copper, which becomes more evident following the addition of O₂ or O₂ plus CH₄ (results not shown).

Effects of mb on the cupric signal in the $g_{\perp}$ region

One surprising result in the examination of membrane samples was the absence of the normally well-resolved superhyperfine structure in the $g_{\perp}$ region associated with the type II Cu(II) of the pMMO (Nguyen *et al.*, 1996; Yuan *et al.*, 1997, 1998a, b, 1999) [Fig. 5b, traces (i) and (v)]. This phenomenon was only observed in membrane preparations from cells cultured in high-copper, i.e. 80 μ M CuSO₄ medium, which has been shown to copper-saturate *Methylococcus capsulatus* Bath (Choi *et al.*, 2003; Zahn & DiSpirito, 1996). To examine whether Cu-mb was responsible for the loss of superhyperfine structure associated with the pMMO, the effect of mb at different copper-to-mb ratios on the EPR spectra from washed membrane samples was examined. Fig. 6(a) shows the EPR spectra of the washed membrane sample from cells cultured in 80 μ M CuSO₄ medium. In these membrane samples, the type II Cu(II) superhyperfine structure was initially well-resolved (Fig. 6a, no-addition trace). However, the addition of mb containing copper-to-mb ratios above 0.25 Cu per mb resulted in the loss of copper superhyperfine structure, as well as the free-radical signal, at $g=2.005$. At copper-to-mb ratios above 0.25 Cu per mb, signal intensity increased in the cupric spectral region and was similar to Cu(II)-titration experiments with purified mb. Fig. 6(b) shows the EPR spectra of washed membrane samples from cells cultured in 60 μ M CuSO₄, where maximal expression of the pMMO has been observed (Choi *et al.*, 2003; Zahn & DiSpirito, 1996). In these membrane samples, the addition of mb with higher Cu(II)-to-mb ratios decreased, but did not eliminate, the resolution of the superhyperfine structure in the $g_{\perp}$ region. The results suggest that the loss of cupric superhyperfine structure in some membrane preparations was the result of the high Cu-mb concentrations. We speculate that the decrease in the resolution of the superhyperfine structure resulted from reduction and/or presence of multiple signals in this region. Increased resolution of the superhyperfine signal following the removal of Cu-mb has also been observed in purified pMMO preparations (Choi *et al.*, 2003).

Fig. 6. X-band EPR spectra at 77 K of the cupric site of the washed membrane fraction from *Methylococcus capsulatus* Bath cultured in 80 μ M (a) and 60 μ M (b) CuSO₄ media. Samples were first incubated with or without the addition of mb containing different copper-to-mb molar ratios, followed by the addition of NADH, O₂ and CH₄. Instrument conditions were identical to those in Fig. 5. Note: $\times$ refers to the amount of signal amplification for presentation purposes.

DISCUSSION

Studies on the association of mb or Cu-mb with the pMMO had been limited, as the only positive correlation between these two molecules was the irreversible loss of methane-oxidation activity following dissociation of these two molecules (Choi *et al.*, 2003; Zahn & DiSpirito, 1996). However, previous studies were performed by using purification procedures involving one or more steps at pH values of 4.0 or lower (Zahn & DiSpirito, 1996), which is now known to destabilize the molecule (Kim *et al.*, 2004). In addition, no precautions were taken for possible photo-degradation. Furthermore, previous attempts at stimulation of pMMO activity in cell-free fractions added mb and Cu(II) separately for control purposes. In this study, mb samples were mixed with copper and incubated for a

minimum of 5 min before addition to reaction mixtures containing pMMO. Initially, this difference would appear trivial, but considering the inhibitory effects of mb samples with copper-to-mb ratios below 0.6 Cu per mb, this difference in addition methodology becomes significant. Another difference between this and previous studies was the higher methane-oxidation activities in the washed membrane fractions. The methane-oxidation activity in the washed membrane samples from previous studies was <20% of the methane-oxidation activity in the samples used in this report. Lastly, the inhibitory effects of mb, with copper-to-mb molar ratios below 0.6 Cu per mb, on methane oxidation by the pMMO also demonstrate the importance of Cu(II)-to-mb ratio in the final sample. The inhibition in samples with copper-to-mb ratios below 0.6 Cu per mb may be due to the removal of copper or other metal(s), such as Fe, from membrane samples.

The EPR and kinetic experiments described here suggest that Cu–mb is a redox-active chromopeptide that stimulates methane oxidation by pMMO. This stimulation was equal to or greater than that observed with Cu(II) and without the toxicity observed at higher Cu(II) concentrations. The stimulation of pMMO activity was similar to the stimulation observed previously with P-centre inhibitors of the bc_1 complex, such as mixothiazol and stigmatellin (Brand & Trumpower, 1994; Choi *et al.*, 2003; DiSpirito *et al.*, 2004; Matsumo-Yagi & Hatefi, 1999; Zahn & DiSpirito, 1996; Zhang *et al.*, 1998). Stimulation of pMMO activity by mixothiazol and stigmatellin has been interpreted as resulting from either the preferential shuttling of electrons to the pMMO or that the pMMO has a quinone- or semiquinone-binding site (DiSpirito *et al.*, 2004). Most of the available evidence supports the second interpretation (Basu *et al.*, 2003; Choi *et al.*, 2003; DiSpirito *et al.*, 2004; Shiemke *et al.*, 1995, 2004; Zahn & DiSpirito, 1996).

The EPR studies presented here suggest interactions between Cu–mb and the type II Cu(II) centre of the pMMO and Cu–mb. The results also suggest that Cu–mb is the probable source of the variability, complexity and controversy associated with the copper centres of the pMMO (Basu *et al.*, 2003; Chan *et al.*, 2004; Choi *et al.*, 2003; Lieberman *et al.*, 2003; Lieberman & Rosenzweig, 2004, 2005; Nguyen *et al.*, 1994, 1996, 1998; Takeguchi & Okura, 2000; Takeguchi *et al.*, 1999; Téllez *et al.*, 1998; Yuan *et al.*, 1997, 1998a, b, 1999; Zahn & DiSpirito, 1996). Laboratories examining the pMMO have differed in the analysis of the EPR spectra. Results from studies using the washed membrane fraction from *Methylomicrobium album* BG8 (Yuan *et al.*, 1997, 1998a, b, 1999) have suggested that the main, if not sole, source of the EPR spectrum is from a type II Cu(II) site. Other laboratories examining pMMO in *Methylococcus capsulatus* Bath have suggested that the spectrum associated with the pMMO is the sum of two EPR signals, one from a type II Cu(II) site and a second either associated with Cu–mb (Choi *et al.*, 2003; Zahn & DiSpirito, 1996) or from a trinuclear Cu(II) cluster (Chan *et al.*, 2004; Nguyen *et al.*,

1994, 1996, 1998). mb from *Methylomicrobium album* BG8 has also been isolated recently (D. W. Choi, Y. S. Young & A. A. DiSpirito, unpublished results). However, the concentrations of Cu–mb in the washed membrane fractions from *Methylomicrobium album* BG8, cultured in NMS medium containing 5–10 μ M CuSO₄, were <10% of the concentration observed in the membrane fractions from either *Methylococcus capsulatus* Bath or *Methylosinus tri-chosporium* OB3b^T. The lower concentration of Cu–mb in *Methylomicrobium album* BG8 may account for the less-complex and better-resolved superhyperfine structure in the $g_{\perp}$ region in samples, as previous studies have shown an increased resolution of superhyperfine structure in the $g_{\perp}$ region following separation of Cu–mb from the $\alpha\beta\gamma$ subunits of the pMMO from this organism (Choi *et al.*, 2003). Taken together, the results support the view that the site of the second EPR signal is from Cu–mb.

In conclusion, the results presented here provide the first direct evidence for the role of Cu–mb in methane oxidation by the pMMO. The exact mechanism of stimulation is still unknown, but the results suggest that Cu–mb increases electron flow to the type II Cu(II) centre(s) in the pMMO and may be involved in radical formation. Cu–mb may also have a secondary role in protection against oxygen radicals and/or delivery of copper to the pMMO, as speculated earlier (Choi *et al.*, 2003; DiSpirito *et al.*, 2004).

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