Methanobactin and the membrane-associated methane monooxygenase in methanotrophy: a tale of two proteins

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Methanobactin and the membrane-associated methane monooxygenase in methanotrophy: A tale of two proteins

by

DongWon Choi

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CHAPTER 1: GENERAL INTRODUCTION

Modified from a book chapter “Respiration in Methanotrophs” published in Respiration in Archaea and Bacteria\(^1\)

Alan A. DiSpirito, Ryan C. Kunz, DongWon Choi, and James A. Zahn

Methanotrophs are Gram-negative bacteria characterized by the utilization of methane or methanol as a sole carbon and energy source. Two general categories of methanotrophs have been identified, Type I and Type II, based on several characteristics including the pattern of internal membranes, carbon assimilation pathway, and predominant fatty acid chain length (1, 6, 25). Methanotrophs play a key role in the global carbon cycle, and may be a significant sink for atmospheric methane (7, 18, 23). In addition to their ecological significance, the potential use of these microorganisms in bioremediation and biotransformations processes has provided incentive for the biochemical characterization of methanotrophs (4, 6, 10, 12).

The oxidation of methane to carbon dioxide by methanotrophs involves a series of two electron steps with methanol, formaldehyde, and formate as intermediates (Fig. 1) (1, 6). The first enzyme in this pathway, the methane monooxygenase (MMO) catalyzes the energy-dependent oxidation of methane to methanol. In some methanotrophs, methane is oxidized to methanol by two different methane monooxygenases (MMOs), a membrane-associated MMO or particulate MMO (pMMO) and a soluble cytoplasmic MMO (sMMO) depending on the copper concentration during growth (19, 20). In cells cultured under a

low copper-to-biomass ratio, the sMMO is predominately expressed, with low, but
detectable levels of pMMO expression (12, 16, 17, 20, 26). Cells cultured under higher
copper-to-biomass ratios express the pMMO exclusively, with no detectable expression
of sMMO (12, 16, 21). While sMMO is a well-characterized enzyme that consists of a
hydroxylase component composed of three polypeptides and a hydroxo-bridged binuclear
iron cluster, an NADH-dependent reductase component composed of one polypeptide
containing both FAD and [Fe$_2$S$_2$] cofactors, and a regulatory polypeptide (4, 6, 8, 19),
information on the molecular properties of the pMMO is limited due to the instability of
the pMMO in cell free fractions.

Purification of the pMMO has been reported from *Methylococcus capsulatus* Bath
(2, 14, 26) and *Methylosinus trichosporium* OB3b (11, 22). The reporting laboratories
agree that the pMMO is a copper-containing enzyme, composed of three polypeptides
with molecular masses of approximately 45,000 (α-subunit), 26,000 (β-subunit), and
23,000 Da (γ-subunit) with a (αβγ)$_3$ molecular structure (5). However, researchers in the
field disagree on the number and type of metal centers associated with the pMMO as well
as the nature of the physiological electron donor. One model proposes the pMMO as a 8
– 15 Cu and 2 Fe-enzyme. In this model, two type II copper atoms and two EPR-silent
iron atoms are associated with the αβγ complex (26). The remaining 6 to 13 copper
atoms are bound to a small, 1,238 Da copper binding peptide/compound (cbc) – now
called methanobactin for its similarity with pyoverdin class siderophores (fig. 2). The
role of copper containing-methanobactin (Cu-mb) is not known; it may be involved in
electron flow to the active site or serve a secondary role such as copper acquisition,
maintaining a particular redox state, protection against oxygen radicals, or as a copper
chaperone. The second theory proposes a 15 to 21 copper enzyme in which the coppers are coordinated into 5 - 7 spin-coupled trinuclear copper clusters, where 2 - 3 clusters are catalytic and 3 - 4 clusters are involved in electron transfer from NADH to the catalytic centers(13-15). The third model proposes the pMMO as a 2 copper and 1 - 2 iron enzyme(2, 24). The first and third models also propose that the pMMO is linked to the electron transfer chain at the quinone level (2, 3, 26) while the second theory proposes the enzyme utilizes NADH as the physiological reductant (14).

One of the main limitations in all of the above models is the use of low activity preparations in the characterization of this novel enzyme. The reported purified preparations show activities ≤ 17 nmol•min⁻¹•mg protein⁻¹, representing 0.1 to 2% of the physiological rates. Recent attempts by Basu et al. (2) have resulted in partially purified preparations with activities in the 50 nmol•min⁻¹•mg protein⁻¹ range, but were unable to purify an active form of the enzyme. This dissertation presents further insight into the biophysical and biochemical characteristics of pMMO and methanobactin using improved purification procedure, which will contribute to elucidate their physiological functions. This research also presents numbers of unusual in vitro activities of methanobactin that may or may not directly related to its physiological functions but could be useful in other applications.

**Thesis organization**

Following the introduction, chapter 2 describes an improved purification procedure resulting in high activity membrane-associated methane monooxygenase (pMMO) preparation along with the growth conditions to stabilize cell-free pMMO
activity. This manuscript also examines the effect of detergent concentration on the metal composition of the prepared pMMO, as well as the issue of the physiological reductant of the pMMO. This work was published in Journal of Bacteriology\textsuperscript{1}. I conducted all organism cultivation, enzyme preparation with different detergent to protein ratios, cell and protein enumeration, activity assays, electrophoresis, immunoblot analysis, metal quantifications, and sample preparations for further analysis. Ryan Kunz and Eric Boyd performed immunoblot analysis, fatty acid analysis, and metals quantifications. Ryan Kunz also involved in the enzyme purification. JongIn Han from Dr. Jeremy Samrau laboratory (Ann Arbor, Michigan) examined sMMO and pMMO expression levels throughout chemostat cell cultivation process. Dr. William Antholine (Milwaukee, Wisconsin) conducted electron paramagnetic resonance (EPR) spectroscopy analysis. Dr. Arlene de la Mora (Ames, Iowa) examined statistical relevancy of the changes of cell physiology during chemostat.

Chapter 3 presents the first direct evidence for the role of copper bound methanobactin (Cu-mb) in methane oxidation by the pMMO. This work was published in Microbiology\textsuperscript{2}. I did cell cultivation, high activity membrane fraction preparation, methanobactin (mb) isolation, enzyme activity assays, protein determination, UV-visible spectroscopy, and sample preparations for EPR analysis. Young Do and Clint Kisting determined solution molecular mass and solid molecular mass of mb. Dr. William Antholine (Milwaukee, Wisconsin) performed EPR analysis.

\textsuperscript{1}Journal of Bacteriology (2003) 185, 5755-5764. Copyright 2003 American Society for Microbiology.
\textsuperscript{2}Microbiology (2005) 151, 3417-3426. Copyright 2005 Society for General Microbiology.
Chapter 4 describes the isolation of low-copper-containing mb samples allowing the first time examination of metal binding and solution properties of mb, and thus its potential role as a chalkopore. This work was published in Biochemistry\(^1\). I conducted cell cultivation, mb isolation, UV-visible absorption spectroscopy, and fluorescence measurements. Clint Kisting and myself prepared EDTA treated mb. Corbin Zea from Dr. Nicola Pohl laboratory (Ames, Iowa) and myself performed isothermal titration calorimetry (ITC). Young Do and myself were responsible for circular dichroism (CD) spectra measurements and analysis. The kinetic measurements of copper binding using stopped-flow reactor was performed by two research groups in parallel; Dr. Mark Hargraove and myself and Peter Shaft from Dr. Scott Hartsel laboratory (Eau-Claire, Wisconsin). X-ray photoelectron spectroscopy (XPS) was also conducted by two research groups in parallel; Eric Boyd from Dr. Gill Geesey laboratory (Bozeman, Montana) and Damon Campbell, Vinay Rao from Dr. Marcus McEllistrem laboratory (Eau-Clarie, Wisconsin).

Chapter 5 presents the spectral and thermodynamic properties of Ag(I), Au(III), Co(II), Cd(II), Fe(III), Hg(II), Mn(II), Ni(II), Pb(II), U(VI), and Zn(II) bindings with special attention given to the coordination and oxidation states of metals. This work was published in Journal of Inorganic Biochemistry\(^2\). I did cell cultivation, mb isolation, metal saturated mb sample preparation, UV-visible absorption spectroscopy, fluorescent spectra measurements, gold nanoparticle production for transmission electron microscopy analysis, and also sample preparations for further analysis. Young Do and myself were

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responsible for circular dichroism (CD) spectra measurements and analysis. Corbin Zea from Dr. Nicola Pohl laboratory (Ames, Iowa) and myself conducted ITC. X-ray photoelectron spectroscopy (XPS) was conducted by two research groups in parallel; Eric Boyd from Dr. Gill Geesey laboratory (Bozeman, Montana) and Lori Scardino from Dr. Marcus McEllistrem laboratory (Eau-Clarie, Wisconsin). Metal quantification of mb was performed by SungWoo Lee from Dr. Jeremy Semrau laboratory (Ann Arbor, Michigan). Dr. William Antholine (Milwaukee, Wisconsin) performed EPR analysis.

Chapter 6 presents the potential secondary roles of Cu-mb in methane catalysis by the pMMO. The oxidase, superoxide dismutase, and hydrogen peroxide reductase activites of mb were examined and compared to the effect of mb on methane catalysis by the pMMO. This work will be submitted to Journal of Inorganic Biochemistry. I did cell cultivation, high activity membrane fraction preparation, mb isolation, UV-visible absorption spectroscopy, all activity assays, and also sample preparations for further analysis. Dr. Marcus McEllistrem laboratory (Eau Claire, Wisconsin) was responsible for molecular mass determination of different mbs and XPS. Metal quantification of mb was performed by Dr. Jeremy Semrau laboratory (Ann Arbor, Michigan). Dr. William Antholine (Milwaukee, Wisconsin) performed EPR analysis.

Chapter 7 proposes that the pMMO has sMMO type dirion catalytic center. The Mössbauer parameters of high activity pMMO were examined and compared with the parameters of sMMO. This work has been submitted to Journal of American Chemical Society. I did cell cultivation, high activity pMMO preparation, UV-Visible absorption spectroscopy, all activity assays, and also sample preparations for further analysis. Dr. Eckard Münck laboratory (Pittsburgh, Pensylvania) was responsible for the Mössbauer
spectroscopy studies. Dr. William Antholine (Milwaukee, Wisconsin) performed EPR analysis.

References


Figure 1. Proposed pathways of methane oxidation in *M. capsulatus* Bath. Membrane associated proteins are shown above the carbon oxidation steps, soluble proteins are shown below the carbon oxidation steps. Abbreviations: Cyt, cytochrome; D-FalDH, dye-linked formaldehyde dehydrogenase; FDH, formate dehydrogenase; MDH, methanol dehydrogenase; N-FalDH, NAD(P)-linked formaldehyde dehydrogenase; pMMO, particulate methane monooxygenase; Q-8, ubiquinone-8; RuMP, ribulose monophosphate; sMMO, soluble methane monooxygenase.

Figure 2. A. Schematic drawing of copper-bound mb adapted from Choi *et al.* (Biochemistry, 2006). B. Crystal structure of mb modified from deposited crystal structure at the Cambridge Crystallographic Data Center, allocated deposit number CCDC241254.
CHAPTER 2: THE MEMBRANE-ASSOCIATED METHANE MONOOXYGENASE (pMMO) AND pMMO-NADH: QUINONE OXIDOREDUCTASE COMPLEX FROM *Methylococcus capsulatus* Bath

A paper published in Journal of Bacteriology\(^1\)

Dong-W. Choi, Ryan C. Kunz, Eric S. Boyd, Jeremy D. Semrau, William E. Antholine, J.-I. Han, James A. Zahn, Jeffrey M. Boyd, Arlene M. de la Mora, and Alan A. DiSpirito

Abstract

Improvements to the purification of the membrane-associated methane monooxygenase (pMMO) have resulted in preparations of pMMO with activities more representative of physiological rates, i.e., > 130 nmol•min\(^{-1}\)•mg protein\(^{-1}\). Altered culture and assay conditions, optimization of detergent to protein ratio, and simplification of the purification procedure were responsible for the higher activity preparations. Changes in the culture conditions focused on the rate of copper addition. To document the physiological events that occur during copper additions, cultures were initiated in media with cells expressing soluble methane monooxygenase (sMMO), then monitored for morphological changes, copper acquisition, fatty acid concentration, as well as pMMO and sMMO expression as the copper concentration was increased from 0

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amended copper (approximately 0.2 µM) to 95 µM amended copper. The results demonstrate that copper not only regulates the metabolic switch between the two methane monooxygenases, it also regulates the level of expression of the pMMO and the development of internal membranes. With respect to stabilization of cell free pMMO activity, the highest cell free pMMO activity was observed when copper addition exceeded maximal pMMO expression. Optimization of detergent to protein ratios and simplification of the purification procedure also contributed to the higher activity levels in purified pMMO preparations. Lastly, the addition of the type-2 NADH:quinone oxidoreductase (NDH) from *M. capsulatus* Bath, along with NADH and duroquinol to enzyme assays increased the activity of purified preparations. The NDH and NADH were added to maintain a high duroquinol to duroquinone ratio.

**Introduction**

Methanotrophs are a group of gram-negative bacteria that utilize methane or methanol as a sole source of carbon and energy (2, 14). The initial oxidation of methane to methanol is catalyzed by the methane monooxygenase (MMO). In some methanotrophs, two different MMOs can be expressed; a soluble cytoplasmic MMO (sMMO) and a membrane-associated or particulate MMO (pMMO), depending on the copper concentration during growth (8, 29, 30). In cells cultured under low copper-to-biomass ratios, ≤0.9 nmol Cu/mg cell protein, the sMMO is expressed (14, 21). Cells cultured under higher copper-to-biomass ratios express the pMMO, with no detectable sMMO expression (27, 34). While sMMO is a well-characterized enzyme that consists of
a hydroxylase component composed of three polypeptides and a hydroxo-bridged binuclear iron cluster, an NADH-dependent reductase component composed of one polypeptide containing both FAD and [Fe₂S₂] cofactors, and a regulatory polypeptide (12, 19, 20, 23, 37), information on the molecular properties of the pMMO is limited due to the instability of the pMMO in cell free fractions.

Purification of the pMMO has been reported from *M. capsulatus* Bath (3, 16, 25, 43) and *M. trichosporium* OB3b (35). The reporting laboratories agree that the pMMO is a copper-containing enzyme, composed of three polypeptides with molecular masses of approximately 45,000 (α-subunit), 26,000 (β-subunit), and 23,000 Da (γ-subunit) with a \((αβγ)_2\) molecular structure (16). However, researchers in the field disagree on the number and type of metal centers associated with the pMMO as well as the nature of the physiological electron donor. One model proposes the pMMO as a 10 – 15 Cu and 2 Fe-enzyme. In this model, two type II copper atoms and two EPR-silent iron atoms are associated with the \(αβγ\) complex (43). The remaining 8 to 13 copper atoms are bound to a small, 1,238 Da copper binding peptide/compound (cbc) that co-purifies with the pMMO (11, 43). The role of copper containing-cbc (Cu-cbc) is not known; it may be involved in electron flow to the active site or serve a secondary role such as copper acquisition, maintaining a particular redox state, protection against oxygen radicals, or as a copper chaperone. The second theory proposes a 15 to 21 copper enzyme in which the coppers are coordinated into 5 - 7 spin-coupled trinuclear copper clusters, where 2 - 3 clusters are catalytic and 3 - 4 clusters are involved in electron transfer from NADH to the catalytic centers (24-26). The third model proposes the pMMO as a 2 copper and 1 - 2 iron enzyme (3, 16, 36). The first and third models also propose that the pMMO is
linked to the electron transfer chain at the quinone level (3, 8, 10, 16, 43) while the second theory proposes the enzyme utilizes NADH as the physiological reductant (25).

One of the main limitations in all of the above models is the use of low activity preparations in the characterization of this novel enzyme. The reported purified preparations show activities ≤ 17 nmol min⁻¹ mg protein⁻¹, representing 2 to 5% of the physiological rates. Recent attempts by Basu et al. (3) have resulted in partially purified preparations with activities in the 50 nmol min⁻¹ mg protein⁻¹ range, but were unable to purify an active form of the enzyme. This paper describes an improved purification procedure, resulting in purified preparations with activities > 130 nmol min⁻¹ mg protein⁻¹. This report examines the growth conditions resulting in the stabilization of cell free pMMO activity, the effect of detergent concentration on the metal composition of the pMMO and also addresses the issue of the physiological reductant of the pMMO.

**Materials and Methods**

**Organism and cultivation.**

*M. capsulatus* Bath cultured for enzyme isolations were grown in nitrate mineral salts media (NMS) with 5 µM CuSO₄ and a vitamin mixture (18) at 42°C in shake flasks under an atmosphere of 30% methane, and 70% air (vol/vol) to an optical density at 600 nm (OD₆₀₀nm) of 1.5-2.0. One liter of flask culture was used to inoculate 2 l of media in a 14 l BioFlo fermentor (New Brunswick, Edison, NJ). Cells were cultured in the fermentor at 42°C and sparged at flow rates between 180 and 200 ml min⁻¹ methane and between 800 and 1,200 ml min⁻¹ of air. The pH of the chemostat was maintained at 7.0
using potassium phosphate monobasic and sodium phosphate dibasic. When the culture reached an optical density at 600 nm (OD$_{600}$) of 1.8 to 2.0, the concentration of copper (added as 500 µM CuSO$_4$ stock solution) and iron (added as a 100 µM NaFeEDTA stock solution) in the culture medium was increased continuously at a rate of 1.6 ± 0.2 µM Cu/Fe/h while maintaining an OD$_{600}$ of 1.8 to 2.0. The media feed rate was set to double the culture volume every 10 to 12 h. Media and copper addition rate were adjusted to maintain a constant cell density between 1.8 and 2.0. Cells were harvested by centrifugation at 14,000 x g for 15 min at 4°C and resuspended in 10 mM 3-[[N-morpholino]propanesulfonic acid (MOPS) (pH 7.3) buffer followed by subsequent centrifugation at 14,000 x g for 15 min. Washed cells were resuspended in 30mM MOPS (pH 7.3), 1 mM benzamidine buffer.

M. capsulatus Bath cultured to monitor the effect of copper addition during growth was cultured as described above with the following modifications. All glassware was acid washed in 0.1N HNO$_3$ and copper was omitted from the media in flask cultures as well as in the initial fermentation media. Culture samples were taken before the addition of copper, 0 µM copper, and when the copper concentration in the chemostat reached approximately 1, 5, 6, 15, 20, 25, 35, 45, 60, 70, 75, 80, and 95 µM amended copper, and were subsequently harvested and washed as described above.

**Enzyme Activity.**

MMO activity was determined by the epoxidation of propylene as previously described (9). The reductants used were NADH (7mM) and/or duroquinol (approximately 30mM) for cell free extracts and formate (2.5 mM) for whole-cell
samples. The reduction of duroquinone to duroquinol was performed as described by Shiemke (31). Both the duroquinol and NADH were lyophilized to remove residual ethanol. Following lyophilization, both reductants were checked for the presence of ethanol on a SRI 8610C gas chromatography system (SRI Instruments, Las Vegas NV) equipped with a flame ionization detector and an 8 x 0.085 in HaySep D column. Additional lyophilization steps were added if ethanol was detected. In reaction mixtures containing NADH dehydrogenase (NDH), approximately 12 µg of NDH, which would reduce 1 µmol duroquinone•min⁻¹ (7), was added. For optimal propylene oxidation rates, 0.4 ± 0.2 mol Cu per mol αβγ pMMO subunit was added to reaction mixtures containing purified pMMO or purified NDH-pMMO complex. The exact concentration of copper added was determined empirically for each sample. All reactions were initiated by the addition of 2 ml propylene and 2 ml of air. Reaction mixtures were incubated at 42°C on a rotary shaker at 250 rpm. In addition to propylene oxidation activity in the soluble fraction, sMMO activity was monitored by the formation of napthol from naphthalene as described by Brusseau et al. (5).

**Isolation of membranes and soluble fractions.**

All manipulations were performed at 4°C under anaerobic conditions (95% argon and 5% hydrogen, v/v). DNAase I (1µg/ml) was added to the washed cell suspension, then deoxygenated by 3-5 cycles of vacuum followed by purging with oxygen-free argon. Prepared cells were lysed with 3 passes on a constant flow French pressure cell at 18,000lb/in². The cell lysate was centrifuged at 14,000 x g for 15 min to remove unlysed cells and cell debris. The supernatant was taken and centrifuged at 150,000 x g for 1 h to
sediment membranes. The membranes were resuspended using a Dounce homogenizer in 30mM MOPS (pH 7.3), 1 M KCl, 1 mM benzamidine buffer and centrifuged for 1 h at 150,000 x g. The washed membrane pellet was resuspended in a minimal volume of 30 mM MOPS (pH 7.3), 1mM benzamidine buffer.

**Solubilization of pMMO.**

A 10% (wt/vol) solution of dodecyl β-D-maltoside was added to the washed membrane fraction to final concentrations of 0.25, 0.5, 0.75 1.0, 1.25, 1.5, 1.75, 2.0, 3.0, and 4.0 g of dodecyl β-D-maltoside per g protein, stirred for 1 h, then centrifuged at 150,000 x g for 1 h at 4°C. Propylene oxidation activity was determined before centrifugation and in the particulate and soluble fractions following centrifugation.

**Purification of pMMO, NDH, and NDH-pMMO complex.**

The detergent solubilized fraction following centrifugation of the 1.2 g dodecyl β-D-maltoside per g membrane protein suspension was loaded on a 5.0 x 7 cm DEAE-Sepharose FF column equilibrated with 30 mM MOPS (pH 7.3), 1 mM benzamidine, 0.1% dodecyl-β-D-maltoside buffer. The column was washed with 30 mM MOPS (pH 7.3), 100 mM KCl, 1 mM benzamidine, 0.1% dodecyl-β-D-maltoside buffer. The NDH-pMMO complex remained bound to the column and eluted with 30 mM MOPS (pH 7.3), 250 mM KCl, 1 mM benzamidine, 0.1% dodecyl-β-D-maltoside buffer. For separation of NDH and pMMO, samples from the DEAE-Sepharose FF column were diluted with and equal volume of 30 mM MOPS (pH 7.3), 1 mM benzamidine, 0.1% dodecyl-β-D-maltoside buffer and loaded on a 2.6 x 20 cm DEAE-Sepharose Fast Flow column
equilibrated with 30 mM MOPS (pH 7.3), 125 mM KCl, 1 mM benzamidine, 0.1% dodecyl-β-D-maltoside buffer. The loaded column was washed with one column volume of 30mM MOPS (pH 7.3), 125 mM KCl, 1 mM benzamidine, 0.1% dodecyl-β-D-maltoside buffer and eluted using 30 mM MOPS (pH 7.3), 250 mM KCl, 1 mM benzamidine, 0.1% dodecyl-β-D-maltoside buffer. The fraction containing pMMO con was then concentrated under nitrogen gas (99.99%) by ultrafiltration using a YM-50 filter (Millipore Corp., Bedford, MA). NDH was also isolated as previously described by Cook and Shiemke (7).

**Dodecyl β-D maltoside treatment of pMMO.**

The dodecyl β-D-maltoside concentration in purified pMMO samples was increased to 0.1, 0.2, 0.3, 0.4 or 0.5 mg dodecyl β-D-maltoside per mg protein. The samples were then incubated under anaerobic conditions for 30 min and applied to a 2.6 x 55 cm Superdex 30 column equilibrated with 30 mM MOPS (pH 7.3), 1 mM benzamidine, 0.1% dodecyl-β-D-maltoside buffer. The pMMO samples were collected from the void volume and the copper containing copper-binding-cofactor (Cu-cbc) collected from the included volume.

**Electrophoresis and immunoblot analysis.**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on pre-casted 10 or 12% Bis-Tris gels using MOPS, or on pre-casted 10 or 12% 2-(morpholino) ethane sulfonic acid (MES)-sodium dodecyl sulfate running buffer as specified by the manufacturer (Invitrogen, Carlsbad, CA) or on SDS- PAGE by the
method of Laemmli (15), gels were stained for total protein with Coomassie brilliant blue R or blotted for immunoassays. Densitometry was performed using a Bioimaging Technologies Biovedeo MP1000 gel documentation system.

Proteins were blotted onto polyvinylidene difluoride-plus transfer membranes (Micron Separations Inc., Westboro, MA.) using an Xcell II Blot Module (Invirogen) according to manufacturer specifications unless otherwise noted. For transfer of the α-subunit of the pMMO the concentration of methanol in the transfer buffer was reduced to 5% and the concentration of sodium dodecyl sulfate increased to 0.02%. Transfer time for the α-subunit of the pMMO was 90 min at 30V while the transfer time for the β- and γ-subunits was 60 min at 30V. Following treatment with serum raised against purified protein, antibodies were detected using the Opti-4CN Substrate Kit according to the manufacturer (Bio-Rad Laboratories, Hercules, CA.).

**Preparation of antibodies against the pMMO.**

Antiserum against pMMO was raised in one New Zealand White rabbit by Animal Pharm Services, Inc. (Healdsburg, CA). Immunoglobulin G was purified from the serum using a Protein A Sepharose CL-4B column as specified by manufacturer (Amersham Biosciences Corp., Piscataway, NJ.).

**Protein, cell count, and metal determinations.**

Samples were assayed for protein by the method of Lowry et al. (22) using bovine serum albumin as a standard. Cells were enumerated on a Beckman Coulter Epics XL Flow Cytometer (Beckman Coulter, Inc., Allendale, NJ).
Sample preparation for metal analysis were determined as previously described (43) and analyzed for copper or iron either by inductively coupled plasma atomic emission-mass spectroscopy (ICP-MS) with a model 4500 ICP-MS (Hewlett-Packard, St.Paul, MN) or with a Perkin-Elmer 1100B Atomic Absorption Spectrophotometer (Sheldon, CT).

The percentage of copper bound to copper binding compound (cbc) in the spent media was determined by the ratio of copper that bound to a Dianion HP-20 column (Supelco, Bellefonte, PA) versus the copper in the void volume from the Dianion HP-20 column. The spent media was loaded on either a 1.5 x 7 cm, a 2 x 11 cm or a 7 x 20 cm Diaion HP-20 column depending of the sample size. The column was washed with 2 column volumes of H2O and the organic phase was eluted from the Dianion HP-20 columns with 50% methanol: 50% acetonitrile (vol:vol). The organic phase consisted of approximately 80% cbc, 12% protoheme IX, and 8% trace proteins and pyrroloquinoline quinone. The elutant containing the cbc fraction was rotary evaporated at 50°C, freeze-dried and resuspended in 2 mM ammonium phosphate buffer (pH 7.0). For the percentage of copper bound to cbc, the concentration of copper was determined in the spent media, in the void volume from the Dianion HP-20 column (soluble copper) and in the fraction that bound to the Dianion HP-20 column (copper bound to cbc).

**Fatty Acid analysis.**

Whole cell samples were normalized to two differ protein concentrations, 1.8 and 3.6 mg/ml protein, for each cell suspension. The cell suspensions were saponified, methylated, and extracted using the described methods of MIDI Corporation (Newark,
DE). In addition to the standards obtained from Microbial ID, Incorporated (Newark, DE), quantification standards were obtained from Supelco, Supelco™ 37 component FAME Mix (Bellefonte, PA) and prepared in hexane. FAME samples were separated using an Agilent 6890 gas chromatograph and identified using the Microbial Identification System (version 4.0) from the MIDI Corporation.

**Amino acid analysis and sequence.**

Amino acid sequence analysis was performed by Edman degradation with an Applied Biosystems 477A protein sequencer coupled to a 120A analyzer. Sequence analysis was performed on samples electroblotted to polyvinylidene difluoride membranes as described above.

**Spectroscopy.**

Optical absorption spectroscopy and X-band electron paramagnetic resonance (EPR) spectra were obtained as previously described (39, 43).

**Total RNA extraction from pure cultures.**

Care was taken in handling RNA to prevent RNA degradation by RNAase as described earlier (13). Briefly, RNAase activity was removed through oven-baking and the use of either RNAase-free compounds or diethyl pyrocarbonate (DEPC, Sigma)-treated water, or treated with DEPC. RNA extractions were performed using QIAGEN total RNeasy kit (QIAGEN Inc., Valencia, CA) and cells lysed by bead-beating. After being treated with RNAase-free DNase I (Promega), total RNA was extracted with QIAGEN total RNeasy kit.
Homologous internal RNA standards were designed with similar base sequences that could be amplified with the same primers used for target mRNA and used for competitive RT-PCR using the QIAGEN OneStep RT-PCR kit. The RT-PCR reaction was carried out in 50µl consisting of 5µl of standard RNA, 5µl total RNA, 10µl 5xRT-PCR buffer, 10µl 5xQ solution, 400µM each of dNTPs, 0.6µM of each primer, and 2µl of QIAGEN OneStep RT-PCR enzyme. The sequences of steps to reverse transcribe and amplify both targets and standards were as follows. Following a RT incubation at 50°C for 30min and heating to 95°C for 15min, 30 PCR cycles with the following amplification profile were conducted; 94°C for 1 min; 55°C for 1min; and 72°C for 1min. All samples were finally extended at 72°C for 10min. PCR products were analyzed quantitatively by capillary electrophoresis on a Beckman P/ACE MDQ instrument (Beckman Instruments, Palo Alto, CA,USA).

**Results**

**Effect of copper and iron in the culture medium on pMMO activity.**

A key step in the initial stabilization and isolation of the pMMO was the cultivation of cells under high, i.e., toxic copper conditions (43). Since the initial isolation, pMMO isolation procedures have used a variety of methods to maintain a high copper to cell density ratio during growth (3, 16, 25, 35). To optimize the stabilizing effect of medium copper on cell-free pMMO activity, a series of continuous and discontinuous copper addition schemes were attempted. The optimal copper addition rate was determined to be 1.7 µM/h in chemostats where the generation time was approximately
10 h and the optical density at 600nm ($A_{600}$) of the culture was 1.9 ± 0.1. Under these culture conditions, whole cell propylene oxidation rates increased with increasing copper concentration up to approximately 24 µM amended copper (Fig. 1). At concentrations between 24 and 80 µM amended copper, whole cell propylene oxidation rates per mg protein showed a slight decrease, followed by an accelerated decrease at copper concentrations above 80 µM. In contrast to whole cell propylene oxidation rates, propylene oxidation activity in the washed membrane fraction was barely detectable below 24 µM amended copper, but increased linearly between 20 to 80 µM amended copper. At higher copper concentrations, pMMO activity in the membrane fraction also decreased, although the decrease in the rate was small compared to the decreased rates observed in whole cells.

For high activity pMMO preparations, iron was also in at required in an iron to copper ratio of 1 to 5. Only low activity, i.e. $\leq 7 \text{nmol}\cdot\text{min}^{-1}\cdot\text{mg protein}$, were obtained in the absence of supplemental iron.

**Effect of copper in the culture medium on pMMO expression and membrane development.**

To document the physiological events that occur during growth under these culture conditions, the initial media copper concentration was changed from 5µM to 0 µM, thus allowing the cells to express the sMMO. As the copper concentration was increased in these chemostats, samples were taken periodically and analyzed for: (a) morphology (b) copper concentration in the spent media, whole cell, soluble and membrane fractions, (c) propylene oxidation activity in whole cell, soluble and
membrane fractions, (d) SDS-polyacrylamide gel electrophoresis of whole cell and soluble and membrane fractions, (e) immunoblots using antibodies against pMMO and methanol dehydrogenase (MDH), (f) transcript levels of the genes encoding polypeptides for both sMMO and pMMO using RT-PCR and capillary electrophoresis, and (g) fatty acid concentration, via MIDI-FAME analysis. The fatty acid concentration was used as an estimate of phospholipid concentration.

Figure 2 shows copper acquisition in cells cultured in increasing copper conditions. Under the above culture conditions the copper concentration in the spent media was constant 0.8 ± 0.3 µM until the copper addition reached 59 µM and then increased at higher concentrations. Of the copper recovered in the spent media less than 4% was associated with the organic fraction, i.e., cbc fraction in the spent media. Between 1 and 20 µM amended copper, the copper associated with the whole cell and membrane fractions increased proportionately with copper addition (Table 1). Between 20 and 60 µM copper addition, the copper concentration in the spend media remained 0.8 ± 0.3 µM, but shows a less than expected increase in copper concentration per mg cell or membrane protein (Fig. 2A). Above 60µM copper, copper accumulated in the spent media and the copper concentration per mg protein in the whole cell and membrane fraction decreased. The results was surprising considering the average copper mass balances of the cell fractionation studies were close to 70% (68 ± 12%) and the copper mass balances of the whole cell (i.e. whole cell and spent media fractions) component of the studies was close to 80% (78 ± 16%). The discrepancy in the between the copper lost from the spent media and cell associated copper was resolved when the cell associated copper was calculated on a per cell number basis and not via protein concentration (Fig.
When plotted against cell number the results show the cell associated copper increased until the amended copper reached 80 µM.

The results from Fig. 2 suggest the presence of highly expressed copper regulated protein(s) in *M. capsulatus* Bath. The pMMO was the obvious candidate for this copper regulated protein and the concentration of both pMMO polypeptides (Fig. 3) and transcript (Fig. 4) were monitored. The peptide and transcript concentrations per cell both demonstrated that the expression levels of pMMO continued to increase even after the switchover from expression of the sMMO to pMMO. Between 1 and 59 µM added copper, the concentration of pMMO polypeptides increases proportionally with copper (Figs. 3 and 4, Table 1). At copper concentrations above 59 µM, the a decrease in the concentration of pMMO polypeptides per cell is observed. Comparison of cells expressing pMMO cultured under 1 and 59 µM copper showed a 15 fold increase in the three pMMO polypeptides, an 18 fold increase in pMMO transcript concentration, and a 2.6 fold increase in the concentration of fatty acids per cell.

Previous results from this and S. Chan’s laboratory have shown that on a per mg membrane protein basis the concentration of pMMO per mg membrane protein was essentially constant with increasing copper concentrations (24, 28, 43). To resolve the difference between the above results and previous studies, ultrastructural examination (Fig. 5) and fatty acid concentrations per cell (Fig. 6) were determined. Fatty acid concentrations per cell were used in an indicator of membrane density. Both studies demonstrated that a direct relationship exists between membrane content per cell and pMMO peptide concentration (*P* = 0.928, *r* = 0.01), pMMO transcript concentration (*P* = 1.00), and the concentration of copper in the culture medium (Table 1). The results also
demonstrate that maximal membrane development and pMMO concentration per cell occurs at approximately 60 µM Cu (Figs. 5 and 6, Table 1). Between 1 and 59 µM amended copper, the percent increase in pMMO to percent increased membrane content per cell was approximately 1 to 0.17. Previous studies have shown a relationship between copper and membrane development in methanotrophs (4, 6, 8, 28 – 30). However, these studies compared only a few copper concentrations, with 10 µM amended copper as the maximum concentration examined.

In addition to the polypeptides associated with the two MMOs, polypeptides with molecular masses of 36,000, 20,000, and 14,000 Da also appear to be regulated by copper (Fig. 2). N-terminal sequencing of all three polypeptides show the 20,000 and 14,000 Da polypeptides are breakdown products of the \( \alpha \)-subunit of the pMMO. The 36,000 Da polypeptide was a mixture of a breakdown product of the pMMO and type 2 NADH:quinone oxidoreductase (NDH (7).

**Detergent to protein ratio.**

Previous studies on the solubilization of the pMMO using dodecyl \( \beta \)-D maltoside focused on the activity in the detergent-solublized fraction, with an optimal detergent to protein ratio of 1.6 mg dodecyl \( \beta \)-D maltoside per mg protein (32, 43). With the higher activity membrane preparations described above, the optimal detergent to protein was re-examined. In contrast to previous studies, the propylene oxidation activity was measured before centrifugation and in the particulate and the detergent solublized fractions following centrifugation. The results are shown in Fig. 7. Two basic conclusions were obtained from the results of the detergent series. First, the optimal detergent to
membrane protein ratio for solublization of propylene oxidation activity was 1 to 1.25 mg dodecyl β-D-maltoside per mg protein. Above 0.3 mg dodecyl β-D-maltoside/mg membrane protein, solubilization of the αβγ polypeptides of the pMMO was proportional and increased with increasing detergent concentrations with maximal solubilization at 4.6 mg of dodecyl β-D-maltoside/mg membrane protein (Fig. 7). However, no activity was observed at detergent to protein ratios above 2.0. Second, both low and high detergent concentrations resulted in the irreversible inactivation of the pMMO. This inactivation was observed within 15 minutes of detergent addition.

To examine the effect of dodecyl β-D-maltoside concentration on pMMO, the pMMO solubilized at different detergent concentrations were purified (Fig. 8). As expected from the results presented in Fig. 7, the pMMO isolated from both low (i.e. 0.5 and 0.75 mg dodecyl β-D-maltoside per mg membrane protein) and high (i.e. 2.0 and 3.0 mg dodecyl β-D-maltoside per mg membrane protein) detergent extractions were inactive. The protein profile of the pMMO purified following each detergent extraction was identical, however the metal composition of the samples varied. The metal content of inactive pMMO isolated from both low and high detergent extractions was approximately 2 copper and 2 iron atoms per αβγ subunit. In contrast, the pMMO isolated following 1.0, 1.2, 1.4, 1.6, mg dodecyl β-D-maltoside per mg of membrane protein extractions were active and contained 10 - 15 copper atoms and 2 iron atoms per αβγ subunit.

**Effect of detergent concentration on purified pMMO.**

To examine the relationship between detergent and metal content of pMMO further, the effect of increasing detergent concentration on purified pMMO samples was
tested. The addition of 0.1 and 0.2 mg dodecyl β-D-maltoside per mg protein had no effect the purified sample. The pMMO retained activity and the copper and iron content remained the same (Fig. 8). However, at concentrations ≥ 0.3 mg dodecyl β-D-maltoside per mg pMMO, complete enzyme inactivation was observed and the copper concentration per pMMO αβγ subunit was approximately 2. The EPR spectra of the purified pMMO sample containing 10 copper before (Fig. 9, trace A) and after (Fig. 9, trace B) the 0.3 mg dodecyl β-D-maltoside treatment in which the copper concentration was reduced to two copper were essentially identical. The spectra from both samples showed the type 2 Cu(II) signals associated with the αβγ subunits of the pMMO were present and of similar intensities (3, 16, 17, 38-41, 43). As in previous studies using Triton X-100, the copper dissociated from the αβγ-polypeptides, was associated with the cbc (43). Over 70% of the copper bound by cbc are Cu(I) and thus EPR silent (11, 43), which explains the similarities in the spectra before and after detergent treatments.

**Purification of pMMO and NDH-pMMO complex.**

The purification of the pMMO and NDH-pMMO complex from *M. capsulatus* Bath were performed as described in the Materials and Methods section and are summarized in Table 2 and Fig. 10. The major loss of activity is observed during cell lysis. The loss of activity during cell lysis and in the steps involved in the isolation of the membrane fraction can be minimized if the procedure is carried out under anaerobic conditions. Recent studies have suggested pMMO activity is not oxygen sensitive when in the membrane fraction (3, 16, 35). However, the pMMO activity in the washed membrane fraction in studies reporting the absence of oxygen sensitivity in the washed
membrane fraction were less than 15% of the activities reported in this study. In washed membrane fractions with propylene oxidation activity below 40 nmol propylene oxidized•min⁻¹•mg protein⁻¹, the activity was stable following 3 h exposure to air. However, in washed membrane fractions with propylene oxidation activity above 40 nmol propylene oxidized•min⁻¹•mg protein⁻¹ complete loss of activity was often observed following exposure to air for 3 h. Following detergent solubilization, all pMMO samples tested were oxygen sensitive. A modest increase in activity is observed at each purification step after detergent solubilization with a final propylene oxidation activities of 147 ± 43 and 134 ± 36 nmol propylene oxidized•min⁻¹•mg protein⁻¹ for the NDH-pMMO complex and purified pMMO, respectively.

**Physiological reductant.**

The initial isolation of the pMMO utilized duroquinol as a reductant (43). Propylene oxidation activity in the pre-column steps was higher if NADH was used as a reductant, however, NADH driven propylene oxidation activity was lost in the final purification steps. Thus, duroquinol is used as the reductant in cell free assays, in spite of the fact that the concentration of duroquinol in the reaction mixtures was below saturation (43). In an attempt to increase the concentration of reduced duroquinone in reaction mixtures, NADH and NDH from *M. capsulatus* Bath were added along with duroquinol to pMMO activity assays. Cook and Shiemke (7) have shown that NDH reduced duroquinone with a $V_{\text{max}}$ of 93 µmol duroquinone reduced •min⁻¹•mg protein⁻¹. The additions of NADH and NDH to pMMO assays resulted in a 20 to 35% increase in enzyme activity (Table 2). The purification procedure for the pMMO was also modified
to co-purify the pMMO with the NDH. In the current purification procedure the concentration of NDH peptide to pMMO is low and the NADH dehydrogenase activity in the NDH-pMMO complex represents less than 80% of the pMMO activity. Thus, this fraction still requires the addition of NDH for optimal activity.

**Discussion**

In addition to the stabilization of cell free pMMO activity, the results presented in this paper demonstrate that copper not only regulates a metabolic switch between the two methane monooxygenases, it also regulates the level of expression of the pMMO as well as the concentration of internal membranes. Previous studies have shown this relationship, however, only a few copper concentrations were examined, and the maximal expression levels were never determined (4, 6, 8, 23, 29, 30, 33). The results presented here show the optimal expression of the pMMO is observed in media containing approximately 60 µM copper, when the cell density is $1.9 \pm 0.3 \times 10^9$ cells/ml. However, for optimal cell free pMMO activity the concentration of copper in the culture medium was increased to 80 µM. Above 60 µM amended copper, the concentration of pMMO per cell is essentially constant, but the copper concentration per cell continues to increase. This increase in copper concentration per cell probably results from the increased saturation of the copper sites in the pMMO fraction. As observed in other laboratories, copper often stimulates the propylene oxidation activity in cell-free and purified pMMO samples (3, 16, 25, 35). An increase in the type 2-Cu(II) signal is also observed following the addition of copper to purified pMMO preparations (1). Again the results
suggest that the copper sites in cellular as well as purified preparations of pMMO are rarely saturated.

In addition to growth conditions, the results reported here illustrate the importance of detergent to protein ratios during pMMO solublization and purification. The inactivation of the pMMO at detergent to membrane protein ratios ≤ 0.75 or ≥ 2.0 apparently resulted from the differential solublization of the pMMO and copper containing cbc (Cu-cbc). At detergent to membrane protein ≤ 0.75, the αβγ polypeptides solublized are Cu-cbc free. Detergent to membrane protein ratios ≥ 2.0 results is the dissociation of the αβγ polypeptides and Cu-cbc. The results suggest the minimal metal content for active purified pMMO as described here is 8 – 10 Cu and 2 iron atoms with 2 Cu and 2 Fe atoms associated with the αβγ polypeptides and 6 - 8 atoms associated with cbc. The role of Cu-cbc in methane oxidation by the pMMO is still in question, but the results indicate Cu-cbc stabilizes pMMO in cell free fractions. This observation differs from two recent reports by Basu et al. (3) and Lieberman et al. (16), which presented partially purified and purified preparation of pMMO containing 2 Cu and 1 to 2 Fe atoms per αβγ subunit. The difference in metal composition of the pMMO in these studies and the results reported here can not be resolved at this time.

The nature of the physiological reductant to the pMMO has been a source of controversy. Most of the available data from this and other laboratories indicates that the pMMO is coupled to the electron transport chain at the cytochrome bc₁ complex probably via the quinone (3, 8, 10, 16, 35, 42, 43) although NADH has also been proposed as the electron donor (25). The reports of NADH-driven pMMO activity are probably due to contaminating NDH in pMMO preparations. The NDH-pMMO complex did show
activity with NADH as a sole reductant. However, the activity was only 30% of the activity following the addition of duroquinol. Thus, duroquinol-free activity may have resulted from contaminating ubiquinone in pMMO preparations. Trace contamination of the cytochrome \(bc_1\) complex has been shown to be a common trace contaminant in pMMO preparations (25, 43). The ability to separate the NADH dehydrogenase and methane (as measured by propylene) oxidation activity indicates the previous reports using NADH as a reductant in pMMO assays were probably due to the presence of NDH in these preparations (25).

**Acknowledgement**

This work was supported by the Department of Energy grant 02-96ER20237 (to AADS) and National Science Foundation grant 9708557 (JDS).

**References**


Table 1. Bivariate correlations of copper concentration in the culture media to methane oxidation activity and concentrations of copper, pMMO peptide and transcript, and fatty acids per cell.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Fraction</th>
<th>Copper Concentration</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 – 19 µM</td>
<td>20 – 59 µM</td>
<td>60 – 80 µM</td>
<td></td>
</tr>
<tr>
<td>Methane Oxidation</td>
<td>Whole Cell</td>
<td>0.91**</td>
<td>0.91**</td>
<td>0.416</td>
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</tr>
<tr>
<td></td>
<td>Soluble</td>
<td>-0.342</td>
<td>-0.382</td>
<td>nd(^1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Membrane</td>
<td>0.959**</td>
<td>0.921**</td>
<td>0.957**</td>
<td></td>
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<tr>
<td>Copper</td>
<td>Spent Media</td>
<td>0.754*</td>
<td>0.644</td>
<td>0.898**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Whole Cell</td>
<td>0.766**</td>
<td>0.848**</td>
<td>-0.937</td>
<td></td>
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<tr>
<td></td>
<td>Soluble</td>
<td>0.902**</td>
<td>0.787*</td>
<td>0.983*</td>
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<tr>
<td></td>
<td>Membrane</td>
<td>0.846**</td>
<td>0.867**</td>
<td>-0.258</td>
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<tr>
<td>Peptide</td>
<td>sMMO</td>
<td>-0.78</td>
<td>-0.83</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pMMO</td>
<td>0.93*</td>
<td>0.980**</td>
<td>0.673</td>
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<tr>
<td>Transcript</td>
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<td>-0.568</td>
<td>-0.568</td>
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<tr>
<td></td>
<td>pMMO</td>
<td>0.977*</td>
<td>0.977*</td>
<td>nd</td>
<td></td>
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<tr>
<td>Fatty Acids</td>
<td>14:00</td>
<td>0.871**</td>
<td>0.911**</td>
<td>-0.811</td>
<td></td>
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<tr>
<td></td>
<td>16:1 cis9</td>
<td>0.626</td>
<td>0.898**</td>
<td>-0.917</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16:1 cis11</td>
<td>0.911**</td>
<td>0.838**</td>
<td>-0.473</td>
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</tr>
<tr>
<td></td>
<td>16:0</td>
<td>0.695*</td>
<td>0.872**</td>
<td>-0.993</td>
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<tr>
<td></td>
<td>17:0</td>
<td>0.742*</td>
<td>0.437</td>
<td>-0.132</td>
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<tr>
<td></td>
<td>16:0 3OH</td>
<td>0.616</td>
<td>0.906**</td>
<td>-0.955</td>
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<td></td>
<td>15:0</td>
<td>0.549**</td>
<td>0.670</td>
<td>-0.948</td>
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</tr>
<tr>
<td></td>
<td>Total</td>
<td>0.710*</td>
<td>0.870**</td>
<td>-0.973</td>
<td></td>
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Note: *\(p \leq .05\); **\(p \leq .01\); \(^1\)not determined
Table 2. Purification of pMMO from *M. capsulatus* Bath using duroquinol, NADH, NADH plus duroquinol or duroquinol, NADH and NDH as the reductant(s). The values represent the results from four different preparations.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Formate</th>
<th>Duroquinol</th>
<th>NADH</th>
<th>Duroquinol + NADH</th>
<th>Duroquinol + NADH + NDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Cell</td>
<td>235 ± 68</td>
<td>nd¹</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Washed Membrane</td>
<td>nd</td>
<td>63 ± 23</td>
<td>81 ± 37</td>
<td>78 ± 45</td>
<td>nd</td>
</tr>
<tr>
<td>Dodecyl β-D-maltoside Extract</td>
<td>nd</td>
<td>51 ± 27</td>
<td>25 ± 14</td>
<td>74 ± 21</td>
<td>nd</td>
</tr>
<tr>
<td>DEAE-Sepharose FF</td>
<td>nd</td>
<td>73 ± 34</td>
<td>36 ± 13</td>
<td>101 ± 33</td>
<td>147 ± 43</td>
</tr>
<tr>
<td>DEAE-Sepharose FF</td>
<td>nd</td>
<td>95 ± 31</td>
<td>0</td>
<td>97 ± 32</td>
<td>134 ± 36</td>
</tr>
</tbody>
</table>

¹not determined
Figure 1. Effect of copper concentration in the culture medium on the rates of propylene oxidation in whole cell (Δ), washed membrane (○), and soluble (□) fractions. Rate of copper addition was 1.7 µM•h⁻¹ while the cell density was maintained at an A₆₀₀ between 1.8 and 2.0.

Figure 2. A. Copper concentration in the spent media (Δ), associated with the whole cell fraction (□), associated with membrane fraction (○) of cells cultured in chemostats with increasing copper concentrations. Chemostat conditions and cell samplings were as described in Fig. 1.
Figure 3. Top, SDS-polyacrylamide gel electrophoresis of whole cell samples from *M. capsulatus* Bath. Cell samples where taken before the addition of copper (0.3 µM copper)(lane A), and when the copper concentration in the chemostat reached 1 µM (lane B), 5 µM (lane C), 15 µM (lane D), 25 µM (lane E), 35 µM (lane F), 45 µM (lane G), 55 µM (lane H), 65 µM (lane I), and 75 µM (lane I) copper. Molecular mass standards (Invitrogen Mark 12 standards: 200, 116.3, 97.4, 66.3, 55.4, 36.3, 21.5, 14.4, 6, 3.5, 2.5 kDa) are shown in lane K. Chemostat conditions and cell samplings were as described in Fig. 1. The cell sample in each lane was standardized to 1.3 x 10^8 cells per lane.

Lower, 43 NT, Coomassie stained gel illustrating the protein remaining in the 43 kDa region of a SDS-polyacrylamide gel following blotting for 1 h. Coomassie stained gel following transfer is included because of the poor transfer of the 43 kDa pMMO polypeptide. Immunoblot analysis of *M. capsulatus* Bath cell fractions with antibodies against the α and β subunits of the pMMO and with antibodies against methanol dehydrogenase, MDH. MDH was used as a non-copper regulated protein control.

Arrows on left indicate sMMO hydroxylase polypeptides and on right indicate pMMO polypeptides.
Figure 4. Transcript concentration of *pmoA* (■) and *mmoX* (□) in *M. capsulatus* Bath cultured in media not supplemented with copper and expressing the sMMO, and following the addition of 1, 5, 25, or 55 µM CuSO₄. Chemostat conditions and cell samplings were as described in Fig. 1.

Figure 5. Thin-section transmission electron micrograph of *M. capsulatus* Bath cultured as described in Fig. 1. Chemostat conditions were Cell samples were taken when the added copper concentration in the culture medium reached 5 µM (A), 20 µM (B), 40 µM (C), 60 µM (D), 80 µM (E), and 89µM (F) Cu. Marker bar equals 200 nm.
Figure 6. Effect of copper concentration during growth on low (A) and high (B) concentration fatty acids in *M. capsulatus* Bath. Fatty acids monitored were: 16:0 3-OH FAME (●), 14:0 FAME (○), 17:0 FAME (△); 15:0 FAME (▲); 16:0 (▼); 16:1 cis 9 FAME (■); 16:1 cis 11 (▲). Chemostat conditions and cell samplings were as described in Fig. 1.
Figure 7. Top. Effect of dodecyl β-D-maltoside on membrane-associated propylene oxidation activity in *M. capsulatus* Bath. Rate of propylene oxidation following incubation in dodecyl β-D-maltoside for 1 h under anaerobic conditions before (○) and following (●) centrifugation at 150,000 x g for 90 minutes. Bottom. SDS-polyacrylamide gel electrophoresis of solublized protein (2µl volume) samples following 150,000 x g centrifugation of washed membrane samples incubated for 1 h of membrane samples with 0.3 (B), 0.5 (C), 0.7 (D), 1.0 (E), 1.4 (F), 1.8 (G), 2.0 (H), 3.0 (I), or 4.0 (J) mg dodecyl β-D-maltoside per mg membrane protein.
Figure 8. Effect of dodecyl β-D-maltoside concentration used in the initial solubilization of the pMMO on the propylene oxidation activity (□), and on the copper (○) and iron (△) composition per αβγ subunit of purified pMMO.

Fig. 9. X-band EPR spectra at 77 K of the cupric site in high detergent treated purified active pMMO (79 mU/mg protein) containing 10 Cu and 2 iron atoms per αβγ complex (A) and following detergent treatment and containing 2 copper and 2 iron atoms per αβγ complex. Experimental conditions: modulation frequency, 100 KHz; modulation amplitude, 5G, time constant 100 ms, microwave frequency 9.191 GHz, and microwave power 5.0 mW.
Figure 10. Inhibition of nitroblue tetrazolium reduction in the presence of Cu-cbc.

Figure 11. SDS-polyacrylamide slab gel electrophoresis of fractions during the purification of NDH-pMMO complex and of pMMO from *M. capsulatus* Bath, lane A, whole cell fraction, lane B, washed membrane fraction, lane C, detergent solublized fraction (1.2 g dodecyl β-D-maltoside per g membrane protein), lane D, first DEAE-Sepharose FF eluate, lane E, second DEAE-Sepharose FF eluate.
CHAPTER 3: EFFECT OF METHANOBACTIN ON THE ACTIVITY AND ELECTRON PARAMAGNETIC RESONANCE SPECTRA OF THE MEMBRANE-ASSOCIATED METHANE MONOOXYGENASE IN *Methylococcus capsulatus* Bath

A paper published in Microbiology

Dong W. Choi, William E. Antholine, Young S. Do, Jeremy D. Semrau, Clint J. Kisting, Ryan C. Kunz, Damon Campbell, Vinay Rao, Scott C. Hartsel, and Alan A. DiSpirito

Abstract

Improvements in the purification of both copper-free methanobactin (mb) and copper-containing methanobactin (Cu-mb) (Kim et al. 2004. Science 305, 1612) resulted in Cu-mb 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). Activities approaching 400 nmol·min⁻¹·mg protein⁻¹ mg in the washed membrane were commonly observed following addition of Cu-mb, which represented 50 to 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

1 Microbiology (2005) 151, 3417-3426. Copyright 2005 Society for General Microbiology.
methanobactin nor was it observed when the copper to mb ratio was below 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 methanobactin ratios below 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 less than two min. At Cu(II) to mb ratios between 0.4 - 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 showed coordination by nitrogen only. 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 and absence of reductants (NADH or duroquinol), and substrates (CH₄ and/or O₂). The results indicated 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 represents the first positive evidence of interactions between the pMMO and Cu-mb.

**Introduction**

Methanobactin (mb), previously called copper-binding compound and copper binding-peptide (cbc), 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 fraction from both *M. capsulatus* Bath and *Methylosinus trichosporium* OB3b (Choi *et al.*, 2005a; DiSpirito *et al.*, 1998; Fitch *et al.*, 1993; Kim *et al.*, 2004; Kim *et al.*, 2005; Tellez *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 αβγ subunits of the pMMO were believed to contain two type II, Cu(II) and two EPR silent Fe atoms. Active preparations also contained of 8 to 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 the enzyme is a trimer, (αβγ)3. Each αβγ monomer contained one type II, Cu(II) atom, and two copper atoms that appear to be spin coupled, similar to the CuA 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 derived from the crystallization buffer, since no Zn(II) was associated with the enzyme preparations before crystallization. Further, results from these studies suggested 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 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 requires 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 results 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, since 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., 2002; Choi et al., 2003; Choi et al., 2005a), as well as in the isolation of mb (this report) and Cu-mb (Kim et al., 2005), however, has caused us to reconsider the potential role of mb and Cu-mb in methane oxidation.

**Materials and methods**

**Organisms, culture conditions, and isolation of membrane fractions.**

*Ms. trichosporium* OB3b and *M. capsulatus* Bath were cultured for mb isolation in nitrate minimal salts (NMS) medium containing 0, 0.2 or 1 µM added CuSO$_4$ as previously described (Choi et al., 2003). The initial copper concentration in no added
CuSO₄ NMS media was 0.29 ± 0.04 µM. The cultures were grown in batch mode to an optical density at 600nm (OD₆₀₀nm) between 0.7 and 1.2 prior to harvesting for mb. When the OD₆₀₀ reached the desired optical density, 80% of the fermentor was harvested and replaced with fresh NMS media. *M. capsulatus* Bath was also cultured in NMS media that contained a final CuSO₄ concentration of either 60 or 80 µM as previously described (Choi *et al.*, 2003). Washed membranes from *M. capsulatus* Bath were isolated under anaerobic conditions as described by Choi *et al.* (Choi *et al.*, 2003).

**Isolation of mb.**

Cu-mb and mb were prepared from the spent media of *Ms. trichosporium* OB3b or *M. capsulatus* Bath. For each harvest, the spent medium was centrifuged twice at 9,000 × g for 20 minutes to remove residual cells. At this stage the spent media was either loaded onto a 7 x 20 cm Dianion HP20 column (Supelco, Bellefonte, PA, USA) or stabilized by the addition of copper as described by Kim *et al.* (Kim *et al.*, 2004), except the final concentration of added copper was reduced to 2 mM. The Dianion HP-20 column was washed with two column volumes of H₂O and eluted with 60% methanol:40% water (vol/vol) and lyophilized. At this stage of purification, mb represented over 97% of the material absorbing at 214 or 280nm and no other chromophores with absorption maxima above 280 nm were present. Purity was checked at this stage of the purification by HPLC-chromatography, by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, and by 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-chromatography on a Beckman Gold HPLC system using a SupelcoSil LC-18 (25 cm x 4.6 mm, 5 µm) column at a flow rate of 1.0 ml min\(^{-1}\) of 10 mM sodium phosphate buffer, pH 6.6, (solvent A) and 80% acetonitrile-H\(_2\)O (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.

**Molecular mass determinations.**

Solution molecular mass of mb samples were determined on a Superdex Peptide HR 10/30 column (Pharmacia/LKB, Sweden) equilibrated with MilliQ water, pH 6.8. The column was calibrated using blue dextran, orange G, bradykinin (molecular mass, 1240 Da), rennin substrate (molecular mass 1,759 Da), insulin (molecular mass 5,734 Da), and horse heart cytochrome c (molecular mass 12,500 Da).

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectra were obtained on a Voyager-DE PRO Biospectrometry Workstation 6075 (PerSeptive Biosystems, Inc., Framingham, MA). Analyses were performed in the reflector, positive mode with time delayed extraction (200 ns). Acquisition mass range was typically between 500 to 5000 Da with laser intensities between 1900-2100 intensity units. The matrix solutions used were \(p\)-nitroaniline (Fluka Chemika) 35 mM in a 1:1 mixture of water:ethanol at pH 6.5. Typically, 1 µl of a 5 mg/ml mb solution was diluted 1:10 with \(p\)-nitroaniline matrix. Two µl 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 previously described and measured either in the liquid phase on an SRI 8610C gas chromatography system (SRI Instruments, Las Vegas, NV, USA) equipped with a flame ionization detector and an 8 x 0.085 in HaySepD column or in the gas phase on a Varian 3900 (Varian Corp., Walnut Creek, CA, USA) equipped with a flame ionization detector and a 30m x 0.53mm Supel-Q™ plot column (Bellefonte, PA, USA) (Choi *et al.*, 2003). Isolation of the cell-free fractions from, copper determinations, and protein determinations were carried out as previously described (Choi *et al.*, 2003). In addition to propylene oxidation activity in the soluble fraction, sMMO activity was monitored by the formation of naphthol from naphthalene as described by Brusseau *et al.* (Brusseau *et al.*, 1990).

The effects of mb on pMMO activity were examined in the washed membrane fraction from *Mc. capsulatus* Bath using mb from *Ms. trichosporium* OB3b. The washed membrane fraction from *Mc. capsulatus* Bath was used since procedures for the isolation of membrane fractions with high pMMO activity have only been developed in this species (Basu *et al.*, 2002; Choi *et al.*, 2003; Yu *et al.*, 2003). Mb from *Ms. trichosporium* OB3b used in these studies for a variety of reasons. First, the EPR spectra of Cu-mb from both methanotrophs were identical (Antholine *et al.*, unpublished results). Second, the stimulatory effects were 10 to 20% higher that observed with equimolar concentrations of Cu-mb from *Mc. capsulatus* Bath (results not shown). Third, the yields of mb from the spent media of *Ms. trichosporium* OB3b were generally several fold higher than observed with *Mc capsulatus* Bath (Choi *et al.*, 2003; DiSpirito *et al.*, 1998;
Kim et al., 2005; Zahn & DiSpirito, 1996). Fourth, the mb from Ms. *trichosporium* OB3b is the best characterized mb, structurally (Kim et al., 2004; Kim et al., 2005), spectrally (Choi et al., 2005a; Choi et al., 2005b; DiSpirito et al., 1998; Kim et al., 2005), and thermodynamically (Choi et al., 2005a; Choi et al., 2005b). Lastly, the Cu-mb from *Ms. trichosporium* OB3b is more soluble than Cu-mb samples from *Mc. capsulatus* Bath which tends 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 *M. capsulatus* Bath were prepared for EPR studies in 6 ml amber serum vials in an anaerobic chamber (Coy Laboratory, Grass Lake, MI, USA), using 5% hydrogen, with 95% argon. In samples containing reductants, either NADH or duroquinol, 2.12 nmol of reductant was added per mg of membrane protein. 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 hypo-vials sealed with Teflon coated silicon septa. Substrates, 2ml of O₂, 2ml of CH₄, or 2ml of both, were added with gas tight syringes and the samples 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 determined as previously described (Choi et al., 2003). Kinetic photodiode array spectral series were taken using a micro-volume stopped-flow reaction analyzer (Applied Photophysics, U.K., and SX.18MV). Spectral series were measured at 2.0°C from 275 to 500 nm using a diode array detector with an integration time of 2.56 ms. The mixing chamber had a 1.0 cm pathlength, and a monochromometer 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. (Yuan et al., 1999).

Metal, and protein determinations.

Isolation and cell free reactions, copper determinations, and protein determinations were carried out as previously described (Choi et al., 2003).

Results

Isolation of mb in the absence of copper

The isolation of copper saturated mb (Cu-mb) has recently been reported by Kim et al. ((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 these procedure stimulated pMMO in whole cell and washed membrane fraction (see below). However, the mb samples isolated by this procedure were copper saturated (i.e. 1.2 ± 0.16 Cu per mb) and dialysis against Na₂EDTA (Kim et al., 2004; Kim et al., 2005) only removed approximately 90% of the Cu associated with mb. This dialysis treatment also altered the thermodynamic and spectral properties of the sample (Choi et al., 2005a; Choi et al., 2005b). To obtain low copper containing mb samples, the purification procedure was simplified to to avoid the breakdown products observed in previous purification procedure. A one-column procedure to purify mb from the spent media as described in Material and Methods. Mb samples purified by this procedure represented over 97% of the material absorbing at 214 nm or at 280 nm (Fig. S1 & S2) and contained no other chromophores (Choi et al., 2005a; Choi et al., 2005b). Sample purity was based on the UV-visible absorption spectra, on metal analysis, and on molecular mass determinations on the fractions obtained following separation of the mb fraction from Dianion HP20 by reverse phase HPLC (Fig. S1). The purified mb samples from this procedure contained 0.01 ± 0.002 Cu per mb, 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 since exposure to light resulted in photoinactivation of the sample (Fig. S3).
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 *Mc. capsulatus* Bath are shown in Fig. 1. The cells and membrane fractions used in this component of the study were from *Mc capsulatus* Bath cultured in media with a final copper concentration of either 60 µM CuSO₄, where the expression levels of pMMO are highest, or at 80 µM CuSO₄, which has been shown to saturate the cells with copper (Choi *et al.*, 2003). The copper and of mb concentrations in membrane samples of cells cultured under these conditions were approximately 250 nmol mg protein⁻¹ (Choi *et al.*, 2003) and 150 nmol mb · mg protein⁻¹, respectively. 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. 1). In whole cell samples, the stimulation of pMMO by copper was three to four fold 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 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 75 to 230 nmol min⁻¹mg protein⁻¹ range, before Cu(II) or Cu-mb additions. These propylene oxidation rates are several fold higher than previously reported rates (Basu *et al.*, 2002; Chan *et al.*, 2004; Choi *et al.*, 2003; Lieberman *et al.*, 2003) and are dependent on copper addition rates during growth. Figs.
1B and 1C represent two different membrane preparations showing high and low activity membrane samples. Maintaining anaerobic conditions, elimination of 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 were similar to that observed in whole cell samples except the stimulatory and inhibitory effects were greater (Figs 1B & 1C). 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 percent stimulation of pMMO activity by Cu-mb increased with the initial activity of the membrane preparation.

Several recent experiments have suggested mb may initially bind copper as a homodimer, i.e. as Cu(mb)_2, followed by the binding of a second Cu(II) resulting in a final molar ratio of one copper per mb, Cu-mb (Choi et al., 2005a; Choi et al., 2005b). To examine if the pMMO in the washed membrane fraction responded differently to Cu(mb)_2, the effect of Cu(mb)_2 on pMMO activity was also examined (Fig. 1C). In contrast to Cu-mb which stimulated pMMO activity, Cu(mb)_2 was inhibitory to pMMO activity (Fig. 1C). To further examine this property, the effect 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 below 0.6 copper per mb and stimulatory at concentrations above 0.6 to 0.8 copper per mb (Fig 1D).
**EPR spectra of mb.**

In an attempt to spectrally identify Cu-mb in the washed membrane fraction of methanotrophs, the EPR spectral properties of purified mb was 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 frequency (S-band) confirm 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 \( (g_{ll}=2.23, A_{ll}=185 \text{ G}) \) were broader than usual (Boas, 1984). This indicates more strain in the axial direction than observed from most type II, cupric complexes. These lines in the \( g_{ll} \) region were sharper at a lower microwave frequency (S-band trace in Fig. 2) and broader at higher microwave frequencies (Q-band). At Q-band, the \( g_{ll} \) lines are broad and not detected (not shown). Second, 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 \( g_{ll} \) region. The 1\(^{st}\) 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 \( g_{ll} \) region. Superimposed on the Cu-mb lines were five of six Mn lines and a free radical signal that were not detected at X- or S-band. Q-band spectra contain Mn and free radical signals that were considered background signals. The 1\(^{st}\) harmonic of the Q-band spectrum emphasizes the sharp lines. They are part of the \( g_{ll} \) region from an axial symmetric site and not from \( g_{x} \) for a rhombic site with three g-values \( (g_{x}, g_{y}, g_{z}) \) 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. Since there were at least
10 lines split by 16 G that were resolved and probably more unresolved lines in the spectra, these lines were attributed to superhyperfine 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 and the $g_{||}$ value of 2.23 and the $A_{||}$ 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 the copper associated with mb was predominately Cu(I) and not EPR detectable (Fig. 3 & 4). In copper titration experiments, the mb concentration was 4 mM and CuSO$_4$ additions started at 240 µM (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 less than expected and the residual Cu(II) has $g_{||}$ about 2.20 and $|A_{||}|$ 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 2N2S and 3N1S 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 added copper and the spectra have values of $g_{||} = 2.23$ and $|A_{||}| = 185$ G indicating nitrogen only coordination.

Time course experiments showed all the Cu(II) added was reduced to Cu(I) in less than 10 min (Fig. 4). The Cu(I) associated with mb was stable for hours in oxygenated solutions and required molar excess of hydrogen peroxide to oxidize the Cu(I) to Cu(II).
The Cu(II) remained associated with mb, and can be observed by EPR spectroscopy (Antholine et al. unpublished results). Upon extended incubation with H$_2$O$_2$, an EPR signal for the cupric site was obtained with $g_{||} = 2.23$ and $A_{||} = 185$G, but without the resolved superhyperfine structure, possibly because there was more strain, or multiple cupric sites, or aggregation. In order to prevent free radical reactions close to the cupric site, HCO$_3^-$ was added before H$_2$O$_2$ 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 $g_{||}$ region with $g_{||}=2.20$ and $A_{||}=182$ 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 variability in the spectral characterization of the pMMO, the membranes were isolated and reaction vials 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 50 to 65% reduced and the addition of NADH did not result in additional reduction of the type II, Cu(II) centers associated with the pMMO (Fig. 5A, traces a and b). The inability to reduce the remaining copper centers may represent the physiological resting state of the enzyme or it
may represent the population of inactive enzyme in these preparations. Addition of CH₄
did not change the spectral properties (Fig. 5A, trace c). However, in the presence of O₂
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 d). The addition of methane and
oxygen were similar to the spectra of O₂ alone, but the free radical signal was reduced by
40% (Fig. 5A, trace e). These results suggest that, as in the sMMO (Wallar & Lipscomb,
1996; Wallar & Lipscomb, 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. 5B illustrates one of the EPR
spectral changes observed in the high activity membrane preparations shown in Fig. 5B.
In the presence of a reductant, the addition of 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 b & S4). The near complete reduction of
the type II, Cu(II) signal suggests 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 c). However, the addition of O₂
resulted in the reoxidation of the type II, Cu(II) center(s) of the pMMO and increased the
intensity of the free radical signal at $g = 2.005$ (Fig. 5B, trace d). 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) center(s) without the formation of the radical
signal at $g = 2.005$ (Fig. 5B, trace e).
In other membrane preparations, the reduction of the pMMO following addition of Cu-mb was comparatively small (Figs. 5C & D). In addition, as in the previous example (Fig. 5B) the addition of high concentrations of Cu-mb did not increase the intensity of the Cu(II) signal in the washed membrane sample suggesting the copper associated with mb remained as Cu(I). In these membrane preparations, the intensity of the radical signal became larger following O₂ addition (Figs. 5C & D, trace d). However, in contrast to the membrane series represented in Fig.5B, the addition of CH₄ with O₂ only resulted in partial quenching of the radical signal (Figs. 5C & D, trace e). In these membrane samples, the only change in the EPR spectra following the addition of higher concentrations of Cu-mb was a decrease in the resolution of the superhyperfine structure in the \( g_\perp \) region (Figs. 5C and D).

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., 2002; 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) As with Cu-mb, the addition of NADH and Cu(II) resulted in the partial reduction of the type II, Cu(II) center of the pMMO (Fig. 5F, trace c). Whether the added Cu(II) was subsequently incorporated into existing mb or to some other site in the membrane fraction has not been determined. 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 (Figs. 5E & F).

**Effects of mb on the cupric signal in the \( g_\perp \) region.**

One surprising result in the examination of membrane samples was the lack or 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; Yuan et al., 1998a; Yuan et al., 1998b; Yuan et al., 1999) (Fig. 6B, traces a and e). This phenomena was only observed in membrane preparations from cells cultured in high copper, i.e. 80µM CuSO₄ media which has been shown to copper saturate M. capsulatus Bath (Choi et al., 2003; Zahn & DiSpirito, 1996). To examine if 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 were examined. Fig. 6A shows the EPR spectra of the washed membrane sample from cells cultured in 80µM CuSO₄ media. In these membrane samples, the type II, Cu(II) superhyperfine structure was initially well resolved (Fig. 8A, no addition trace). However, the addition of mb containing copper to mb ratios above 0.25 Cu 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. 6B shows the EPR spectra of washed membrane samples from cells cultured in 60µM 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⊥ region. The results suggest that the loss of cupric superhyperfine structure in some membrane preparations were the result of the high Cu-mb concentrations. We speculate 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).

Discussion

Studies on the association of mb or Cu-mb with the pMMO had been limited since 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 determined using purification procedures involving one or more steps at pH values of 4.0 or less (Zahn & DiSpirito, 1996) which is now known to destabilize the molecule (Kim et al., 2004). In addition, no precautions were taken for possible photodegradation. 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 method 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 were less than 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 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 previously observed with P center inhibitors such as mixothiazol and stigmatellin, of the \( bc_1 \)-complex (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 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., 2002; Choi et al., 2003; DiSpirito et al., 2004; Shiemke, 1995; Shiemke et al., 2004; Zahn & DiSpirito, 1996).

The EPR studies presented here suggest interactions between Cu-mb and the type II, Cu(II) center of the pMMO and that Cu-mb. The results also suggest that Cu-mb is the probable source of the variability, complexity, and controversy associated with the copper centers of the pMMO (Basu et al., 2002; Chan et al., 2004; Choi et al., 2003; Lieberman et al., 2003; Lieberman & Rosenzweig, 2004; Lieberman & Rosenzweig, 2005; Nguyen et al., 1994; Nguyen et al., 1996; Nguyen et al., 1998; Takeguchi, 2000; Takeguchi et al., 1999; Tellez et al., 1998; Yuan et al., 1997; Yuan et al., 1998a; Yuan et al., 1998b; Yuan et al., 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; Yuan et al., 1998a; Yuan et al., 1998b; Yuan et al., 1999) have suggested the main if not sole source of the EPR spectrum is from a type 2, Cu(II) site. Other laboratories examining pMMO in *M. capsulatus* Bath have suggested the spectrum associated with the pMMO is the sum of two EPR signals, one from a type II, Cu(II) site and a second which as been associated with either Cu-mb (Choi et al., 2003; Zahn & DiSpirito, 1996) or from a trinuclear Cu(II) cluster (Chan et al., 2004; Nguyen et al., 1994; Nguyen et al., 1996; Nguyen et al., 1998). Mb from *M. album* BG8 has recently been isolated (Choi et al., unpublished results). However, the concentration of Cu-mb is less than 10% of the concentration observed with either *M. capsulatus* Bath or *M. trichosporium* OB3b (Choi et al., unpublished results). The lower concentration of Cu-mb in *M. album* BG8 may account for the less complex, and better-resolved superhyperfine structure in the $g_\perp$ region in samples from this organism. 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 (Choi et al., 2003). Taken together, the results support the view that the site of the second EPR signal was 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 Cu-mb increases electron flow to the type II, Cu(II) center(s) in the pMMO and many be involved in radical formation. Cu-mb may also have a secondary role in protection against oxygen radicals (Choi et al., 2003) and/or delivery of copper to the pMMO (DiSpirito et al., 2004).
Acknowledgements

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References


Figure 1. Effect of Cu-mb on the rates propylene oxidation by *M. capsulatus* Bath expressing the pMMO. (A) Effect of mb (◇), Cu-mb (●) and copper sulfate (△) on whole-cell propylene oxidation rates in *M. capsulatus* Bath. Cells were cultured in a chemostat to an optical density of 1.2 at 600nm as previously described (Choi et al., 2003) and harvested when the added copper concentration to the media reached 80 µM. Protein concentration in reaction vials were 1.4 mg/ml. (B) Effect of copper sulfate (△) and Cu-mb (●) on propylene oxidation rates in the washed membrane fraction of cells grown and harvested as described in panel A. (C) Effect if copper sulfate (△), Cu-mb (●), and Cu(mb)₂ (υ) on propylene oxidation rates in the washed membrane fraction of cells grown and harvested as described in panel A. (D) The effect of Cu(II) to mb molar ratio on the rate of methane oxidation in the washed membrane fraction from cells cultured in 60 µM Cu(II) (○) and from cells cultured in 80 µM Cu(II) (●). The lines labeled No mb (80 µM) and No mb (60 µM) illustrate the activity in the washed membrane fraction with no added mb.
Figure 2. Q-, S-, and X-band EPR spectra at 77K of Cu-mb. The regions with lines split of 16 G are circled. $A_{ll}$ and $g_{ll}$ are indicated by lines and arrows. Spectrometer conditions: X-band: microwave frequency 9.195 GHz; microwave power 5 mW; temp. 77K; 4 scans averaged. S-band: microwave frequency 3.334 GHz; microwave power 16 dB (incident power 10 mW); temp. -140°C; 4 scans averaged. Q-band: microwave frequency 34.786 GHz; microwave power 26 dB; temp. -140°C; 9 scans averaged. The modulation amplitude was 5 G; scan time 4 min.; and time constant 0.128 sec for all spectra.

Figure 3. X-band EPR spectra at 77 K of mb (concentration 4 mM) following the addition of 0.06, 0.125, 0.25, 0.33, 0.4 and 0.5 molar equivalents of CuSO$_4$. $A_{ll}$ and $g_{ll}$ are indicated by lines and arrows. Experimental conditions: modulation amplitude, 5 G, modulation frequency, 100 KHz, microwave power, 5 mW, temperature 77 K.
Figure 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, 2, 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$_2$O$_2$, the hydrogen peroxide was added to a final concentration of 2.7 or 10 mM H$_2$O$_2$ and incubated for 1, 2, 4, 10, 20, or 30 min at room temperature, the frozen in liquid nitrogen. A$_\parallel$ and g$_\parallel$ are indicated by lines and arrows. Experimental conditions: modulation amplitude, 5 G, modulation frequency, 100 KHz, microwave power, 5 mW, temperature 77 K.
Figure 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 *M. capsulatus* Bath expressing the pMMO isolated under anaerobic conditions. A and B were from the washed membrane samples used in Fig. 5B, and C – F were from washed membrane samples shown in Fig. 5C. A. gain increased by a factor of 5.76 compared to B.

(A) Effect of substrate additions on EPR spectra of the cupric site of the washed membrane fraction from *M. capsulatus* Bath; (a) resting, (b) following the addition of 2.12 nmol NADH per mg membrane protein; (c) following the addition of 2.12 nmol NADH and 1.9 µmol CH₄ per mg membrane protein; (d) following the addition of 2.12 nmol NADH and 1.9 µmol O₂; (e) following the addition of following the addition of 2.12 nmol NADH and 1.9 µmol CH₄ and 1.9 µM O₂ per mg membrane protein.

(B) Effect of Cu-mb and substrate additions on EPR spectra of the cupric site of the washed membrane fraction from *M. capsulatus* Bath; (a) following the addition of 2.12 nmol NADH,
Figure 5. (continued)
(b) following the addition of 23.5 nmol Cu-mb and 2.12 nmol NADH per mg membrane protein;
(c) following the addition of 23.5 nmol Cu-mb, 2.12 nmol NADH and 1.9 µmol CH₄ per mg membrane protein;
(d) following the addition of 23.5 nmol Cu-mb, 2.12 nmol NADH and 1.9 µmol O₂;
(e) following the addition of following the addition of 23.5 nmol Cu-mb, 2.12 nmol NADH, 1.9 µmol. CH₄ and 1.9 µM O₂ per mg membrane protein.

(C) and (D) Effect of Cu-mb concentration on EPR spectra of the cupric site of the washed membrane fraction from *M. capsulatus* Bath.

(C) Effect of 20 nmol Cu-mb per mg membrane protein; (a) resting, (b) following the addition of 2.12 nmol NADH, (c) following the addition of 20 nmol Cu-mb and 2.12 nmol NADH, (d) following the addition of 20 nmol Cu-mb, 2.12 nmol NADH and 1.9 µmol O₂, (e) following the addition of following the addition of 20 nmol Cu-mb, 2.12 nmol NADH, 1.9 µmol. CH₄ and 1.9 µM O₂ per mg membrane protein.

(D) Effect of 70 nmol. Cu-mb per mg membrane protein; (a) resting, (b) following the addition of 70 nmol. Cu-mb, (c) following the addition of 70 nmol Cu-mb and 2.12 nmol NADH, (d) following the addition of 70 nmol Cu-mb, 2.12 nmol NADH and 1.9 µmol O₂, (e) following the addition of following the addition of 70 nmol Cu-mb, 2.12 nmol NADH, 1.9 µmol. CH₄ and 1.9 µM O₂ per mg membrane protein

(E) and (F) Effect of Cu(II) concentration on EPR spectra of the cupric site of the washed membrane fraction from *M. capsulatus* Bath.

(E) Effect of 20 nmol. Cu(II) per mg membrane protein; (a) resting, (b) following the addition of 2.12 nmol NADH, (c) following the addition of 20 nmol Cu(II), 2.12 nmol NADH, and 1.9 µmol O₂, (d) following the addition of following the addition of 20 nmol Cu(II), 2.12 nmol NADH, 1.9 µmol. CH₄ and 1.9 µM O₂ per mg membrane protein.

(F) Effect of 70 nmol Cu(II) per mg membrane protein; (a) resting, (b) following the addition of 70 nmol Cu(II), (c) following the addition of 70 nmol Cu(II) and 2.12 nmol NADH, (d) following the addition of following the addition of 70 nmol Cu(II), 2.12 nmol NADH, 1.9 µmol. CH₄ and 1.9 µM O₂ per mg membrane protein.

Instrument conditions were modulation frequency, 100 kHz; modulation amplitude, 5 G; time constant, 100 ms; microwave frequency, 9.191 GHz; and microwave power, 5.0 mW. Note: X refers to the amount of signal amplification for presentation purposes.
Figure 6. X-band EPR spectra at 77 K of the cupric site of the washed membrane fraction from *M. capsulatus* Bath cultured in 80μM CuSO₄ (A) and 60μM CuSO₄ media. Samples were first incubated with or without the addition of mb containing different copper to mb molar ratios, and following by the addition of NADH, O₂, and CH₄. Instrument conditions were identical to those in Fig. 5. Note: X refers to the amount of signal amplification for presentation purposes.
Supporting Online Material

Supporting Materials and Methods

**UV-visible absorption spectroscopy.** UV-visible absorption spectroscopy was determined as previously described (Choi et al., 2003). Kinetic photodiode array spectral series were taken using a micro-volume stopped-flow reaction analyzer (Applied Photophysics, U.K., and SX.18MV). Spectral series were measured at 2.0°C from 275 to 500 nm using a diode array detector with an integration time of 2.56 ms. The mixing chamber had a 1.0 cm pathlength, and a monochrometer 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).

**Supporting Results and Discussion**

**HPLC-Chromatography of mb samples from Dianion HP-20 column.** Mb samples from Dianion HP-20 columns separated into three mb fractions when chromatographed on reverse phase HPLC columns (Fig. S1). Exception for the associated metals, analysis of the HPLC fractions A- C by UV-visible absorption spectroscopy, ICP-MS and MALDI-TOFF showed the fractions were identical to samples from the Dianion HP-20 column (results not shown). Fractions A and B (Fig. S1) were both spectrally identical to the fraction from the Dianion HP-20 column, i.e. all three contained 0.01 ± 0.002 Cu per mb. Fraction A and B appear to represent the different conformations of mb, possibly monomer and dimer forms as suggested by molecular mass determinations via gel filtration (Fig. S2). The UV-visible absorption spectra,
molecular mass determined by MALDI-TOF mass spectroscopy, and the metal composition of fraction C were identical to copper saturated mb, i.e. $1.2 \pm 0.16$ Cu per mb. Mb apparently removed residual copper from the reverse phase column. Without the addition of Cu(II), additional purification of peaks A and B by HPLC on reverse phase columns resulted in several peaks and a variety of breakdown products as described by Kim *et al.* (Kim *et al.*, 2005).

**Dimer/multimer formation of mb.** Spectral and thermodynamic results suggest mb initially binds Cu(II) as a dimer (Choi *et al.* unpublished results. Homogenous mb samples were examined by gel filtration to determine if mb exists as a dimer or multimer. Mb samples migrated as broad peaks in gel filtration columns (Fig. S2A) with approximate molecular masses between 2,500 and 3,500 Da and between 950 and 1,800 Da, which suggested mb exists in both dimer and monomer forms in aqueous solutions. Both fractions were identical when analyzed by MALDI-TOF mass spectroscopy. When peak fractions labeled mb$_2$ and mb were concentrated and loaded separately on the Superdex Peptide HR 10/30 column, the chromatographs were similar to the original, and migrated as two broad peaks (data not shown). The results suggest in aqueous solutions mb exists in both monomer and dimer forms. The addition of Cu(II) to mb at Cu(II) to mb ratios of 0.5 Cu(II) per mb (0.5 Cu-mb and Cu(mb)$_2$), at 1 Cu(II) per mb (Cu-mb or Cu$_2$mb$_2$ also migrated as two fractions with molecular masses suggesting monomer and dimer forms (Fig. S2A).

The addition of copper to the spent media prior to purification has been shown to stabilize mb (Kim *et al.*, 2004; Kim *et al.*, 2005). To examine the metal binding and
spectral properties of mb Kim et al., (2005), dialyzed Cu-mb against Na₂EDTA to remove the bound copper. However, following dialysis against Na₂EDTA the spectral and thermodynamic properties of the sample suggested molecule does not return to its native non-copper bound form (Choi et al., 2005a; Choi et al., 2005b). To examine if the dialysis treatments altered the mb population into a more structurally homogenous state, mb samples were examined by gel filtration. As shown in Fig. S2B, a comparatively small fraction of the sample migrated with an apparent molecular mass in the dimer range with the remaining material migrating with an apparent molecular mass between 1,000 and 1,500 Da (Fig. S2B). The addition of Cu(II) to Na₂EDTA treated samples resulted in a homogenous sample with a molecular mass of approximately 900 Da (Fig. S2B, Cu-mb). The small size of Cu-mb as measured by gel filtration was not surprising considering the compact structure of this molecule (Kim et al., 2004). The results suggest that mb is a dynamic molecule in aqueous solutions with individual molecules of mb or copper containing mb interacting to form stable dimers and possibly multimers. Dialysis against Na₂EDTA appeared to alter the dynamic nature of mb in aqueous solutions resulting in a more homogeneous sample especially following the addition of Cu(II).

**Photoinactivation of mb.** Following isolation, mb samples were stable if the samples were freeze-dried and stored in the dark at -20°C. Aqueous solutions of mb were also stable if stored in the dark at -80°C and were stable for one freeze-thaw cycle. Exposure to light resulted in photoinactivation of the sample. To examine the photoinactivation of mb, mb samples were rapidly (1.6 ms) mixed at 2.0°C with copper-free deionized water, mb exposed to an unfiltered 150 W xenon lamp and the spectra
monitored every 2.5 ms for 500 s (Fig.S3). Singular value decomposition (SVD) (Henry & Hofrichter, 1992) analysis was used to determine the minimum number of species contributing to the spectral properties of mb during photodecay of mb (Fig. S3B). In this analysis, time dependent absorbance data matrix, A, can be decomposed into an output data matrix where $A = U S V^T$, $V$ being the matrix of the time-dependent amplitudes, $S$ is the singular values of $A$, and matrix $U$ representing the basic spectra of mb at $2.0^\circ$C obtained on a log scale over 500 s. Inspection of the data from Figs. S3A and S3B shows two significantly independent changing species. In this analysis, the first significant basis spectral vector resembled a difference spectra between copper-free mb and Cu-mb (Fig. S3B, panels a & b). The second represented a difference spectrum between this form and the final species, producing a Gaussian band at 394 nm and an additional correlated band evolving at about 280 nm (Figs. S3B, panels c and d). The results show that the major bands at 340 nm and 394 nm are independent chromophores and that the 394 nm chromophore was selectively degraded to a UV absorbing product(s) at 280 nm. The fact that the band was at 394 nm suggests that it is probably the terminal isopropylester associated 4-hydroxy-5-thionyl imidazole group, which has a longer conjugated system. The other 4-hydroxy-5-thionyl imidazole chromophore is likely at 340 nm and was less photolabile. Cu-mb, on the other hand showed very little change under these conditions demonstrating the protective effect of Cu on mb structure (data not shown).

**Free Radical Signal.** The EPR spectrum of the cupric region of washed membrane fraction following addition of Cu(II), Cu-mb and/or O$_2$ showed a signal with a
g-value of 2.005 characteristic of an aromatic free radical (Fig. S4) (Berry et al., 1990; DeGray et al., 1992; Lassmann et al., 1993; Pedersen & Finazzi-Agro, 1993; Whittaker & Whittaker, 1990; Whittaker & Whittaker, 1988). The signal was stable, showed a peak to peak width of 6.7 G, and required low microwave power levels to avoid saturation (results not shown). The radical was very intense following exposure to O₂ and increased with an associated increase in the type II, Cu(II) signal associated with the pMMO. In addition, this radical signal decreased along with the intensity of the type II, Cu(II) associated with the pMMO following CH₄ addition suggesting involvement in methane oxidation. Similar radical signals have been observed in the purified pMMO as well as in the membrane fraction from methanotrophs expressing the pMMO (Basu et al., 2002; Yuan et al., 1998a; Yuan et al., 1998b; Zahn & DiSpirito, 1996).

**Supporting References**


Figure S1. Reverse phase HPLC chromatographic separation of mb fraction from Diaion HP-20 column. Instrument conditions were as follows: Flow rate, 1ml/min; Detector wavelengths, 214nm (-----) and 340nm (………); Column, SupelcoSil LC-18 (25 cm x 4.6 mm); Solvent A, 10 mM sodium phosphate buffer, pH 6.6; Solvent B, 80% acetonitrile: 20% H2O; Gradient, 5 – 35% solvent B over 50 min.

Figure S2. (A). Separation of mb isolated as described in this report on Superdex peptide HR 10/30 column (-----; mb), following the addition of 1.0 Cu per mb (-----; Cu-mb), and following the addition of 0.5 Cu per mb (- - - -; 0.5 Cu-mb). (B) Separation of mb isolated by the procedure of Kim et al. (2004; 2005), and following by dialysis against Na2EDTA, resulting in a sample with 0.1Cu per mb (-----; 0.1 Cu-mb), and followed by the addition of 0.9 Cu(II) per mb to the dialyzed samples (-----; Cu-mb). Abbreviations, V0, void volume, Vl included volume. Column conditions: flow rate 0.9 ml/min; sample volume 0.1 ml; sample 2.4 mg protein.
Figure S3. A. Time-resolved UV-visible absorption spectra of mb exposed to an unfiltered 150 W xenon lamp output for 500 s, spectra were taken every 2.5 ms. B. Singular value decomposition of the data in Figure 3. The data produce two significant basis spectra for which the time-dependent amplitude have large signal to noise ratios. The first (b) results from a decrease in the amplitude of the entire spectrum and the second (d) results primarily from a decrease in the absorption associated with the 4-thionyl-5-hydroxy imidazole group absorbing at 394nm.
Figure S4. X-band EPR spectra at 77 K of the free radical region of the washed membrane fraction of *M. capsulatus* Bath following the addition of Cu-mb (A) and following the addition of Cu-mb, NADH and CH₄ (B). The radical signal at \( g = 2.004 \) on the right was not marked and best seen in Fig. 7A is a background signal. Instrument conditions were modulation frequency, 100 kHz; modulation amplitude, 2 G; time constant, 100 ms; microwave frequency, 9.191 GHz; and microwave power, 16dB.
CHAPTER 4: SPECTRAL, KINETIC, AND THERMODYNAMIC PROPERTIES OF Cu(I)- and Cu(II)-BINDING BY METHANOBACTIN FROM Methylosinus trichosporium OB3b

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Abstract

To examine the potential role of methanobactin (mb) as the extracellular component of a copper acquisition system in Methylosinus trichosporium OB3b, the metal binding properties of mb were examined. Spectral (UV-visible, fluorescence, and circular dichroism), kinetic, and thermodynamic data suggested copper coordination changes at different Cu(II) to mb ratios. Mb appeared to initially bind Cu(II) as a homodimer with a comparatively high copper affinity at Cu(II) to mb ratios below 0.2 Cu(II) per mb, with a binding constant (K) greater than EDTA (log K= 18.8) and an approximate $\Delta G^\circ$ of -47 kcal · mol$^{-1}$. At Cu(II) to mb ratios between 0.2 to 0.45 Cu(II) per mb the K dropped to $2.6 \pm 0.46 \times 10^8$ with a $\Delta G^\circ$ of -11.46 kcal · mol$^{-1}$ followed by

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another $K$ of $1.40 \pm 0.21 \times 10^6$ and a $\Delta G^\circ$ of -8.38 kcal $\cdot$ mol$^{-1}$ at Cu(II) to mb ratios 0.45 to 0.85 Cu(II) per mb. The kinetic and spectral changes also suggested Cu(II) was initially coordinated to the 4-thiocarbonyl-5-hydroxy imidazolate (THI) and possibly Tyr, followed by reduction to Cu(I), and then coordination of Cu(I) to 4-hydroxy-5-thiocarbonyl imidazolate (HTI) resulting in the final coordination of Cu(I) by THI and HTI. The rate constant ($k_{\text{obsI}}$) of Cu(II) binding to THI exceeded that of the stopped flow apparatus used, i.e. $> 640 \text{ s}^{-1}$, whereas the coordination of copper to HTI showed a 6 to 8 ms lag time followed by a $k_{\text{obsII}}$ of $121 \pm 9 \text{ s}^{-1}$. Mb also solublized and bound Cu(I) with a $k_{\text{obsI}}$ to THI $> 640 \text{ s}^{-1}$, but with a slower rate constant to HTI, $k_{\text{obsII}} = 8.27 \pm 0.16 \text{ s}^{-1}$, and appeared to initially bind Cu(I) as a monomer.

**Introduction**

Methanotrophs are characterized by their ability to utilize methane as a sole carbon and energy source (1). These cells are ubiquitous and play a major role in the global cycling of carbon and nitrogen as well as in the degradation of hazardous organic materials (1-5). In contrast to other bacteria, the copper requirement for methanotrophs expressing the membrane associated or particulate methane monoxygenase (pMMO) is several fold higher than the organisms iron requirements, and higher than observed in other aerobic bacteria (1, 6-15). In methanotrophs, copper has been shown to regulate expression of both the soluble and methane-associated methane monoxygenases, membrane development, as well as the expression of several other polypeptides that appear to be involved in either one carbon metabolism, copper regulation or copper
transport (8-11, 15-21). As methanotrophs have a high demand for copper, and copper has pleiotropic effects on methanotrophic physiology, these cells must have an effective mechanism to collect copper. In fact, methanotrophs do appear to have a unique copper uptake system, similar to the siderophore based iron uptake systems, utilizing a novel copper-binding chromopeptide called methanobactin (mb) (11, 15, 22-24).

Methanobactin (mb), is a small 1,154 Da copper-binding chromopeptide initially identified in the methanotroph, Methylococcus capsulatus Bath, during the isolation of the membrane-associated or particulate methane monooxygenase (pMMO) (15, 22-24). Separation of copper-containing mb (Cu-mb) from pMMO results in the irreversible inactivation of the enzyme (9, 15). Cu-mb has been shown to act as an oxygen radical scavenger and also to increase electron flow to the pMMO, but the involvement of Cu-mb in methane oxidation by the pMMO is still in question (8). In addition to the cytoplasmic membrane, mb has also been identified in the spent media of both Mc. capsulatus Bath and the type II methanotroph Methylosinus trichosporium OB3b (15, 22, 25). A number of studies have suggested mb acts as the extracellular component of a copper acquisition system (11, 15, 22-26). Lastly, it has been suggested that mb may serve as a copper chaperone for the pMMO or as a regulatory protein (8-10, 15, 19). Attempts to determine a single or distinct function to mb have proven unsuccessful, and taken together these studies suggest mb may belong to a growing group of proteins known as ‘moonlighting proteins’ (27-32). By definition, moonlighting proteins have the capacity to carry out two or more unrelated functions (28, 30, 32). The physiological functions of different moonlighting proteins vary with changes in cell location, oligomeric state, ligand/substrate concentration, change in physical environment, and/or
complex formation with other proteins. Tompa et al. (32) have suggested that moonlighting proteins are intrinsically unstructured proteins and it is this property that enables these proteins to have distinct functions. Consistent with other moonlighting proteins, gel filtration studies suggest mb will change conformation or form different oligomers at different Cu(II) to mb rations (8).

The crystal structure of copper containing mb (Cu-mb) samples isolated following the exposure to copper concentrations in excess of 1000 Cu(II) per mb has recently been determined (23, 24) (Figure 1). The molecule showed some structural and spectral similarities to siderophores in the pyoverdin class (33, 34). However, mb differed from this group of siderophores in amino acid composition and contained a biologically-unique chromophore, 4-thiocarbonyl-5-hydroxy imidazolate (THI) or 5-hydroxy-4-thiocarbonyl imidazolate (HTI) which were responsible for copper coordination (23, 24). In addition to copper coordination by THI and HTI moieties, the primary structure and amino acid composition of mb shows other potential metal binding sites (35-41). Of particular interest to this study is the tyrosine-pyrrolidine group, which is structurally similar to tyrosine-proline. The phenoxide ion of Tyr has been shown to bind Cu(II), but ionization of Tyr generally only occurs at pH values around 10 (42). However, this ionization has been shown to occur at lower pH values in short peptide chains if Tyr is a terminal amino acid or if Pro is present (1). In mb, pyrrolidine structurally acts as a proline, providing a β-turn in the molecule (24). In addition, as observed in Pro, pyrrolidine does not possess an ionizable nitrogen, which can inhibit Cu(II) coordination by Tyr (40, 43-45). Recent improvements in the isolation of low-copper-containing mb samples (8) allows for the first time examination of metal binding and solution properties of mb, and thus its
potential role as a copper siderophore or chalkophore (24). The results indicate mb is a dynamic molecule in solution and that the initial coordination of Cu(II) differs from the coordination observed in the final sample.

**Materials and Methods**

**Organism, culture conditions, and isolation of methanobactin.**

*Ms. trichosporium* OB3b was cultured in either 0 or 0.2 µM CuSO₄ amended nitrate minimal salts (NMS) medium as previously described (9). Mb, copper containing mb (Cu-mb), and mb isolated following exposure to a 100 fold molar excess of Cu(II) or copper stabilized (Cu-s-mb) were isolated from *Ms. trichosporium* OB3b as described previously (8, 23). Removal of copper from Cu-s-mb required long term (approximately 12 h) dialysis against three changes of 5 mM Na₂EDTA in milliQ water. This dialysis procedure reduced the copper to mb ratio from 1.2 ± 0.1 Cu per mb to 0.1 ± 0.02 Cu per mb and for reference purposes was called EDTA-mb. Mb isolated by the procedure of Choi *et al.* (8) contained 0.01 ± 0.0003 Cu per mb. In an attempt to remove the remaining Cu from mb, 10 ml of 10 mM mb was dialyzed for 12 h against 3 changes of 10 l of 5mM Na₂EDTA dissolved in MilliQ water. However, following dialysis, the copper to mb molar ratio increased to 0.1 ± 0.02 Cu per mb.

**Metal titration of mb and EDTA-mb.**

Metal titration experiments were performed using 50 or 100 µM aqueous solutions of mb or EDTA-mb. Mb and EDTA-mb both buffered the reaction mixtures to
pH 6.8. Stock solutions of either CuSO₄, or CuCl (100 µM, 1 mM, or 10 mM) were used in metal titrations under both aerobic and anaerobic conditions. For anaerobic titrations, solutions first degassed using three vacuum-purge cycles with argon, then incubated for 12 h in an anaerobic chamber (Coy Laboratory, Grass Lake, MI, USA) under an atmosphere of 5% hydrogen and 95% argon. In some samples, divided cuvettes (Optiglass Ltd, Hainault, UK) were used with copper and mb solutions in different chambers. Cuvettes were sealed, removed from the anaerobic chamber, and mixed immediately before analysis. In other samples, mb solutions were added to septum cuvettes (Starna cells Inc., Atascadero, CA) in the anaerobic chamber. The cuvettes were sealed before removal from the chamber, and anaerobic solutions of CuCl or CuSO₄ were added with gas tight syringes. Samples were checked for oxygen contamination using the resazurin based anaerobic indicator strips (Oxoid Ltd, Basingstoke, Hants, England) in the anaerobic chamber and to indicator strips in a closed anaerobic serum vial.

**Spectroscopic measurements.**

UV-visible absorption spectroscopy was determined on either a Cary 50 (Varian Inc., Palo Alto, MA), or an Aminco DW2000 spectrophotometer (SLM Instruments Inc, Urbana, IL).

Fluorescence measurements were determined on a Cary Eclipse Fluorescence spectrophotometer (Varian Inc.). Scan parameters consisted of an excitation slit of 5 nm, an emission slit of 5 nm, and a photomultiplier tube voltage of 600 V. Excitation wavelengths (e.g. 254, 282, 340, and 394 nm) were based on the UV-visible absorption
maxima of metal free mb. CuSO$_4$ and CuCl stock solutions were freshly prepared in MilliQ water, pH 6.8, and kept on ice prior to use. With the exception of time course experiments, all CuCl solutions were mixed between titrations then incubated for 5 min before spectra were taken.

Circular dichroism (CD) spectra were recorded between 180 and 585 nm on a JASCO J-710 spectropolarimeter (Jasco Co., Tokyo, Japan) using a 1.0 mm fused quartz cell. CuSO$_4$ and CuCl stock solutions were prepared as described above for fluorescence measurements.

**X-ray photoelectron spectroscopy (XPS).**

XPS was performed on a Model 5600ci spectrophotometer (Perkin Elmer Inc., Eden Prairie, MN) as previously described (46). The instrument was calibrated at the Au4f$_{7/2}$, Cu2p$_{3/2}$ and Ag3d$_{5/2}$ photopeaks with binding energies of 83.99, 932.66 and 368.27 eV respectively. A 5 eV flood gun was used to offset charge accumulation. A consistent 800 µm spot size was analyzed using a monochromatized AlK$_\alpha$ ($h\nu=1486.6$ eV) X-ray source at 300 W and pass energies of 93.9 eV for survey scans, and either 58.7 or 5.9 eV for high-resolution scans. The system was operated at a base pressure of 10$^{-8}$-10$^{-9}$ torr. An emission angle of 45° was used throughout. Following baseline subtraction, curves were fitted employing combinations of Lorentzian and Gaussian line shapes. Sample charging was accounted for by referencing the principal C1s photopeak to 284.8 eV.

XPS was also performed on a model Phoibos-150 hemispherical analyzer (SPECS Scientific Instruments, Sarasota, FL). The instrument includes a load lock and operates at
a routine base pressure of $2.5 \times 10^{-10}$ torr. The spectrometer was calibrated on Ag$3d_{5/2}$ and C1s from HOPG graphite (Alfa Aesar). Samples were illuminated with Mg$K_{\alpha}$ X-rays ($h\nu = 1253.6$ eV) from a source operated at 200 W. Spectra for C, N, O, S, and Cu were collected using a consistent spot size of 1.2 mm, normal emission, and pass energy of 20 eV. Total illumination time with X-rays was ~1.5 h per sample. Using the C1s HOPG signal and Cu$^{2+}$ signal from pure CuCl$_2$, we determined that the N 1s peak showed no appreciable change in peak shape or binding energy; the broad peak was consistently observed at 399.6 eV, and served as the reference for all spectra collected. Mb was titrated with 30 mM CuCl$_2$ solution, mixed for 1 min, dripped onto a graphite crystal (HOPG), dried under He, and loaded into vacuum. Spectra were fitted to a Shirley background and peaks with a fixed Gaussian to Lorentzian ratio. Cu 2$p_{3/2}$ peaks showed only two states for all samples studied; all Cu peaks had a FWHM of 1.9 eV. S XPS data were fit with two peaks having a FWHM of 1.90 eV. Fitting the data to four peaks to account of spin-orbit splitting of the S 2p state improved the fit but did not change the binding energy shifts or the conclusions. As with Cu, binding energies were corrected for charging using the N 1s peak.

**Isothermal Titration Calorimetry (ITC).**

Isothermal titration calorimetry (ITC) was performed on a Microcal VP-ITC microcalorimeter (Northampton, MA). The instrument was calibrated using the built-in electrical calibration check. All ITC experiments were conducted in water at 25°C and solutions were degassed immediately prior to use. Titrant solutions, Cu(II) (800 µM) as CuSO$_4$ in MilliQ H$_2$O, were added at an interval of 1200 sec for injections 1-23 and 300
sec for injections 24-60 into the stirred cell containing 100 µM mb, with a stirring rate of 550 rpm. To test for sample stability, the UV-visible absorption spectrum of the sample was monitored at 25°C. With the exception of the absorption maxima at 340 and 394 nm the spectrum of mb was stable. The absorption at 340 and 394 nm decreased continuously at a rate of 0.35% per h or 4.2% during a typical titration series (results not shown). No correction was taken for this possible sample loss. To correct for heats of dilution, control experiments were performed in the absence of mb and were subtracted from the titrations containing mb. Between measurements, the sample cell was washed with following manufacturers’ recommendations. The system was then rinsed three times with 100 µM mb. Data was analyzed using nonlinear least squares curve fitting in Origin 7.0 (OriginLab Corp., Northampton, MA).

**Kinetics of copper binding.**

Kinetic measurements of copper binding were made with a 4-syringe Biologic SFM400/S stopped-flow reactor coupled to a MOS 250 spectrophotometer (Bio-Logic Science Instrument SA, Claix, France). This is a 4-syringe system with independent drives for each syringe with minimal dead time of 1.5 ms. Kinetics of copper binding by mb was monitored at 340 or 394 nm. The reaction mixtures contained 50 µM mb, and 25 to 1000 µM CuSO₄. Both reactants were prepared in H₂O and the final pH following mixing was 6.8.

Kinetic measurements were also carried out using a SX.18MV micro-volume stopped-flow reaction analyzer (Applied Photophysics, UK). This is a 2-syringe photodiode array system with a minimal dead time of 1.0 ms. Spectral series were
measured at 2.0 or 20°C from 275 to 500 nm using a diode array detector with an integration time of 2.56 ms. Spectra was monitored every 2.6 ms for 500 s. The reaction mixtures, 50 μM mb and 25 to 250 μM CuSO₄ solutions, were prepared in MilliQ H₂O. The mixing chamber had a 1.0 cm pathlength, and a monochrometer 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. Kinetics of copper binding by at fixed wavelengths was derived from the scan series.

**Metal, thiol, and protein determinations.**

Copper and protein determinations were carried out as previously described \((8)\). The presence of thiol in mb was determined using the thiol and sulfide quantification kit from Molecular Probes, Inc. (Eugene, OR).

**Statistical analysis.**

Pearson correlation coefficients and probabilities were determined by bivariant correlation analysis using SPSS 10.1.3 (SPSS Inc. Chicago, IL).

**Results**

**UV-visible absorption spectra of mb.**

UV-visible absorption spectra in the 200 to 800 nm range of mb showed absorption maxima at 254, 302, 340, and 394 nm (Figure 2; Table 1). The spectra were similar to but not identical to the spectra from copper stabilized mb (Cu-s-mb) samples
following dialysis against Na₂EDTA (EDTA-mb) as described by Kim 

et al. (23). The major differences involved the higher absorption at 340 and 394 nm, and the presence of the small absorption maximum at 302 nm (Figure 2, Table 1). The selective decreases in the absorption maxima at both 340 and 394 nm in mb have been observed with copper binding (Figure 2), photodegradation (8, 23) and sample storage (see Materials and Methods).

Based on the crystal structure of Cu-s-mb (24), the absorption maxima at 340 and 394 nm are believed to be associated with HTI and THI, respectively (23). If this assumption is correct, the absorption maximum at 394 nm can be tentatively assigned to the THI group associated with the terminal isopropylester which has longer conjugated system (Figure 1). The absorption maximum of HTI would then be assigned to the 340 nm. The structure of Cu-s-mb shows copper is coordinated by the \( N^\varepsilon \) atom of each imidazole and the S atom of the two-thiocarbonyl groups (Figure 1). Consistent with this chromophore assignment, Cu(II) addition to mb resulted in a decrease in the absorption maxima at 340 and 394 nm (Figure 2A).

The absorption maxima at 254 nm has been associated with the two Cys moieties of mb (23). Interestingly, the absorbance in the 254 nm region decreased until the copper to mb ratios reached 0.25 Cu(II) per mb suggesting further reduction of Cys population in the sample. Above 0.25 Cu(II) per mb, the absorbance increases continuously suggesting oxidation to form cystine consistent with the crystal structure of Cu-s-mb (24). The absorption changes near 255 nm have also been reported for Cu(II) complexes with deprotonated nitrogens which gives rise to a charge-transfer (CT) absorption in this region (47-49) and with Cys-S-Cu(I) ligand to metal CT (LMCT) (50-54). In the case of
imidazole (Im), the molar absorption coefficient decreased in the 240 – 290 nm region with an increase in the molar ratio of copper to imidazole and the absorption maximum was shifted from 278 nm at 0.013 Cu(II) per Im to shorter wavelengths which at 0.3 Cu(II) per Im molar ratio was 254 nm (47). Thus, the trend in absorption at 254 nm in Cu(II) titration of mb shows some similarity with copper titration of Im. If the absorption changes at 254 nm during Cu(II) titrations resulted from Cys- or thiocarbonyl-S CT the results suggest a copper to metal CT (MLCT) at copper to mb ratios below 0.25 followed by a Cys- or thiocarbonyl-S LMCT at copper to mb ratios above 0.3. The absorbance changes at 254 nm during anaerobic Cu(I) titrations of mb (see below) would be consistent with this S-MLCT/LMCT model.

The absorption maxima at 282 and 302 nm may be associated with phenolic and phenoxide ion forms of tyrosine, respectively (42). Consistent with this hypothesis, the increased absorption at 282 nm was associated with the decreased absorption at 302 nm following copper addition (Figure 2D). The phenolic proton of Tyr residues have been shown to ionize under neutral pH conditions when Tyr is the terminal residue or if the peptide contains a Pro residue (43, 44, 55-57). The Tyr in mb is neither a terminal residue nor does it have a Pro residue, but it does contain a pyrrolidine residue adjacent to the Tyr, which functions structurally as a Pro in the molecule (Figure 1).

Changes in the UV-visible absorption spectra were observed at Cu(II) to mb ratios at or above of 0.001 Cu(II) per mb, or at 50 nM CuSO₄ (Fig. 2 B and C). At lower Cu(II) to mb ratios, no spectral changes were observed following corrections for sample dilutions. At copper to mb molar ratios between 0.001 to 0.4 Cu per mb, the spectral changes at 340 and 394 nm were proportional to the concentration of Cu(II), but little to
no changes were observed at molar ratios above 0.6 Cu per mb. This latter result was inconsistent with previous Cu(II) binding studies (8, 22), and metal analysis on samples used in this study. As in previous studies, exposure of mb to a molar excess of Cu(II) to mb resulted in a 1.2 ± 0.1 Cu to mb ratio (8, 15, 22). Metal analysis following copper titration experiments showed that at molar ratios below 1.1 Cu(II) per mb, essentially all of the added copper was bound to mb. In addition, the titration endpoints were ≥ 0.85 Cu(II) per mb during isothermal titration calorimetry. Lastly, the crystal structure of Cu-mb shows one copper atom per mb (24). Taken together, the UV-visible absorption results suggest mb binds one Cu per mb and initially binds Cu(II) as a homodimer.

**Kinetics of copper binding.**

Cu(II) binding by mb from *Ms. trichosporium* OB3b was too fast to measure when saturating concentrations of Cu(II) were used, i.e. the reaction was complete in less than 2.6 ms at 20°C or 2°C (results not shown). In an attempt to reduce the reaction rate, the concentration of Cu(II) was lowered below saturation. Using this approach, spectral changes at 340 nm could be monitored at 20°C, if the Cu(II) to mb ratios were ≤ 3 Cu(II) per mb. Under these conditions, a 6 to 8 ms lag period was followed by an observed rate constant of $k_{\text{obsII}} = 121 \pm 9 \text{ s}^{-1}$ (Figure 3). However, even at equimolar concentrations, the reaction rate was too fast to measure at 394 nm with stopped flow apparatus used and the rate was estimated at $k_{\text{obsI}} > 640 \text{ s}^{-1}$. Although the kinetic measurements at 340 nm were obtained at sub-saturating Cu(II) concentrations, the results demonstrate the rate of Cu(II) binding by THI occurred before HTI. Absorption changes were observed at 394 nm under these experimental conditions, but were opposite to that observed with copper
binding. Specifically, the absorbance at 394 nm increased during binding to the 340 nm chromophore (Figure 3). The reason for this increased absorption at 394 nm was not determined, but is consistent with a change in copper coordination from THI alone to a coordination involving both THI and HTI.

Consistent with the spectral properties described here and below, the kinetics of Cu(II) binding by EDTA-mb differed from mb. Using EDTA-mb the absorption changes at both 394 and 340 nm appeared identical (i.e. $k_{obs} > 640$ s$^{-1}$) suggesting initial Cu(II) coordination by both THI and HTI (results not shown).

**Fluorescence spectroscopy.**

Mb displayed four emission peaks when excited at wavelengths of 254 nm, 282 nm, 340 nm, and 394 nm (Figure 4A). The characteristic emission peak of Tyr at 310 nm was observed following excitation at 282 nm ($\lambda_{ex282}$) (58, 59). Excitation at 394nm ($\lambda_{ex394}$) resulted in emissions with maxima at 461, 610, and 675 nm and excitation at 340nm ($\lambda_{ex340}$) resulted in a broad emission with a maximum at 461 nm. As expected (60), excitation at 254 nm ($\lambda_{ex254}$) resulted in the Tyr emission peak at 310 nm. Unexpectedly, excitation at 254 nm also resulted in the emission peaks observed following excitation at 340 nm, at 461 and 610 nm (results not shown). These observations were different from the emission spectra recently reported by Kim et al. (23). The first difference involved the intense emission peak at 280 nm following excitation at 282 reported by Kim et al. (23). The intense emission at 280 nm appears to be related to the first harmonics following excitation at 280 nm rather than emission from the Tyr, since the emission was very close to excitation wavelength and too intense to be emissions from Tyr. Tyr is a
comparatively weak fluorophore with typical Stokes shift of ~30 nm (58-62). The intensity of the first harmonics peak would have masked emissions at 310 nm (Figure 4). The second difference was in the emission peaks at 610 and 675 nm following excitation at 394 nm which were not reported by Kim et al. (23). In an attempt to determine the reason for the differences reported here and by Kim et al. (23), EDTA-mb samples were also examined. The emission spectra following excitations at 280, 340 and 394 nm were similar to the results presented in Figure 4 except the emissions at 310, 461, and at 675 nm were only 30 to 85% of intensity observed in mb (Figures 4B - 4D).

The addition of Cu(II) quenched emissions at 310 nm ($\lambda_{ex}254$ or $\lambda_{ex}282$), at 610 nm ($\lambda_{ex}254$, $\lambda_{ex}282$, $\lambda_{ex}340$, or $\lambda_{ex}394$) and at 675 nm ($\lambda_{ex}394$) (Figure 4) while no changes were observed at the broad emission peak 461 nm ($\lambda_{ex}254$, $\lambda_{ex}340$ or $\lambda_{ex}394$). This observation also differs from the recent report by Kim et al. (23) which showed quenching of the emission at 461 nm by Cu(II). In this case, examination of the spectral properties of EDTA-mb did show the difference resulted from the different sample preparations (Figure 4C). The difference in degree of quenching of the emissions at 461 nm was probably due to differences in the initial coordination of Cu(II) by mb and by EDTA-mb. Quenching of the emission from HTI (i.e. emission at 461 nm) would not be expected to occur if Cu(II) was initially coordinated by THI and possibly Tyr, then reduced to Cu(I) before the coordination to HTI. However, if EDTA-mb was in a conformation similar to that observed in the crystal structure of Cu-s-mb (24) (Figure 1) and Cu(II) was initially coordinated by both THI and HTI, quenching of the emissions from HTI (i.e. at 461 nm) should occur along with the quenching of emissions at 610 and
675 nm associated with THI. The results from CD spectra described below suggest this was the case for both mb and EDTA-mb.

**Circular dichroism spectra.**

Like many small polypeptides with disulfide bonds, the CD spectrum of mb is of an unordered protein with a strong negative band at 201 nm and weak bands between 211 and 231 nm (Figure 5A)(63). The CD spectrum of mb also showed weak positive bands at 302 and 364 nm. Following copper addition, the Cu-mb complex showed a decrease in 201 nm along with strong positive bands at 314 nm characteristic of N–Cu charge CT transition and at 406 nm characteristic of phenolate oxygen-Cu CT transition (Figures 5A and 5D) (38, 44, 56, 63, 64). Alternatively, positive band enhancement near 412 nm (1st Cotton effect, THI) and negative band enhancement near 360 nm (2nd Cotton effect, HTI) can be interpreted as exciton coupled spectra of this two chromophore system (65).

In contrast to mb, the CD spectra of EDTA-mb were almost identical to Cu-mb or to Cu-s-mb suggesting mb does not return to its native configuration following removal of Cu via dialysis against Na₂EDTA (Figure 5B and 5E). The largest change in CD-spectra following Cu(II) addition to EDTA-mb was the N–Cu CT transition at 314 nm, but even this change showed little correlation to copper concentrations (results not shown). The results suggest there was a comparatively small change in the secondary structure of Cu-mb and Cu-s-mb samples following removal of copper by dialysis against Na₂EDTA.

**X-ray photoelectron spectroscopy.**

Previous studies have shown that 60 to 100% of the copper bound to mb was cuprous copper (8, 9, 15, 22-24). To address the reason for this variability several
preparations of mb were examined at a variety of Cu(II) concentrations. The results show the percentage of Cu(II) reduced to Cu(I) by mb varied with the copper to mb ratios and decreases from 91 ± 4% at low Cu(II) to mb ratios to approximately 75% at equimolar concentrations of Cu(II) and mb (Figure 6A). The less than 100% reduction of Cu(II) to Cu(I) in reaction mixtures containing low copper concentrations was surprising after examination of higher copper to mb ratios (Figure 6A). The source of the reductants in mb has not been determined. As described above, the spectral changes in the 250 nm range following Cu(II) additions may represent changes in the oxidation state of Cys-S. High-resolution XPS spectra (pass energy = 10 eV) for sulfur from mb were used to examine this possibility (Figure 6B). Fitting the data for metal-free mb revealed two S states: a large peak at 163.3 eV representing 69% of the sulfur signal, and a smaller peak at 161.4 eV (Figure 6B). The major peak at 163.3 mV was assigned to Cys and Met S based on the reported binding energies of 163.6 eV for both Cys (thiol) and Met S (66-68). Formation of a disulfide bridge between two Cys shifts the S binding energy up by approximately 0.5 eV (67). The addition of Cu(II) to mb did not alter the XPS signal at 163.3 eV, and the absence of a new state at energies above 163.3 eV suggests the expected disulfide signal was either below our detection limits or the oxidation state of S on Cys did not change. Attempts to quantify the thiols of mb at the beginning and end of the titration by the method of Singh et al. (69) were negative suggesting the sulfhydryl groups of the two Cys were oxidized in the initial and final sample. These results suggest that the peak at 163.3 eV arises from Met S and Cys as disulfide.
The smaller peak at 161.4 eV was attributed to the thiocarbonyl S (66-68). Studies of thiourea report a S binding energy of 162 eV, which shifts 0.5 to 0.7 eV higher upon binding to Cu. In contrast to thiourea, the thiocarbonyl groups of mb are bond to a hydroxyimidazole group, which extends the local conjugation. The observed binding energy for mb thiocarbonyl S at 161.4 eV reflects this greater conjugation. Binding of mb to Cu shifts the observed binding energy up to 161.9 eV, consistent with results for thiourea-based model compounds binding to Cu (66, 67). The completed binding energy shift of the thiocarbonyl S states at copper to mb ratio of 0.5 Cu per mb indicates that mb initially binds Cu as a dimer.

**Thermodynamic properties of Cu(II) binding by mb.**

Initial isothermal titration calorimetry (ITC) experiments for Cu(II) binding by mb from *Ms. trichosporium* OB3b showed a difference in thermodynamic properties at copper to mb ratios of 0.2 and at 0.45 Cu(II) per mb with a titration endpoint at 0.85 Cu(II) per mb (Figure 7; Table 2). When the titration data in this copper concentration range were analyzed by nonlinear least-squares curve fitting in Origin 7.0 they fit a two site model better than a one binding site model (Table 2). A third high affinity binding constant was also observed at Cu to mb ratios below 0.2 Cu per mb (Figure 7 E & F, Table 2). However, attempts to determine the binding constant at low copper to mb ratios proved difficult. Decreasing titrant concentrations resulted in an unstable baseline due to the small energy change and increasing mb concentrations above 1.8 mM resulted in sample precipitation following copper additions. Measurements between 0.07 and 0.2 Cu per mb provided reasonable results with excellent curve fits with a K of 3.25 x 10^{34} ±
3.0 \times 10^{11} \text{ and a } \Delta G^\circ \text{ of } -47.16 \text{ kcal-mol}^{-1}. \text{ The high K below 0.2 Cu per mb was comparable to the binding constants observed for Fe(III) binding by peptide siderophores (33, 34), but were orders of magnitude higher than expected for copper binding given the structure (40, 45, 48, 57, 70-72) even if mb initially binds as a multimer (73, 74). Given the few titration points, we feel it prudent to estimate the initial K of mb at } > 8 \times 10^{18}. \text{ This binding constant at low Cu to mb ratios was based on the greater affinity of mb over Na}_2\text{EDTA which has a log } K_{\text{EDTA}} = 18.8 \text{ at pH 7.0 for Cu(II) (75) (see Materials and Methods). The complex pattern of Cu(II) binding was also consistent with spectral (UV-visible, fluorescence, CD and EPR), and gel filtration data (8, 22), suggesting a series of subunit interactions and/or conformational changes during Cu(II) binding and reduction.}

\text{ITC was also used to determine if the spectral changes that occur during dialysis against Na}_2\text{EDTA to remove copper from Cu-s-mb samples by dialysis against Na}_2\text{EDTA (EDTA-mb) affected the copper binding properties of the molecule. As shown in Table 2, the initial high affinity copper properties were lost, and the remaining copper binding constants decreased by 1 to 1.5 orders of magnitude. Using the isolation procedure described by Choi et al. (8) the molar ratios of Cu to mb, Cu to Cu-s-mb, and Cu to EDTA-mb were 0.01, 1.2, and 0.1 Cu per mb, respectively. Thus, the initial Cu concentration in EDTA-mb samples could account for the loss of the first high-affinity binding constant, but not for the lower binding constants at copper ratios above 0.1 copper per mb.}

\textbf{Solublization and binding of Cu(I) by mb and EDTA-mb.}

\text{In addition to Cu(II), mb will also bind and solublize Cu(I) under both aerobic and anaerobic conditions (Figures 8 & 9). The UV-visible absorption (Figures 8 & 9),}
fluorescence (results not shown), and CD (Figures 5C and 5F) spectral changes observed during Cu(I) titrations where similar to Cu(II) titrations, except the changes at in the titration trends occurred at approximately 1 Cu(I) per mb suggesting mb bound Cu(I) as a monomer (Figure 8). Also in contrast to Cu(II) (Figure 2), 1.5 to 2.0 Cu(I) per mb was required to saturate the UV-visible absorption changes (Figure 8B). The Cu(I) to mb ratios above 1 Cu(I) per mb suggests mb may have a weak secondary copper-binding site. The spectral changes at 254 nm may represent this secondary binding site (Figure 8) and involve the two Cys moieties or may represent a change from Cys- or thiocarbonyl-S MLCT to Cys- or thiocarbonyl-S LMCT.

The kinetics of Cu(I) binding at 394 nm following Cu(I) addition to mb were also similar to Cu(II), i.e. $k_{\text{obs I}} > 640$ s$^{-1}$. However, the kinetics of binding to HTI (i.e. absorption changes at 340 nm) were significantly slower, $k_{\text{obs II}} = 8.27 \pm 0.16$ s$^{-1}$. The kinetics of Cu(I) binding by mb were similar under aerobic and anaerobic conditions suggesting mb bound Cu(I) before dismutation of Cu(I) to Cu(II) occurred (Figure 9). Since the kinetic differences of $k_{\text{obs II}}$ between Cu(II) and Cu(I) were large, the addition of Cu(II) before coordination of Cu(I) to HTI, was used to determine if Cu(II) could displace mb from the surface of insoluble CuCl (Figure 9B). The addition of Cu(II) after the spectral changes at 394 nm were complete, but before completion of the spectral changes at 340 nm resulted in a small increase in absorption at both 340 and 394 nm, but did not alter the kinetics of Cu(I) binding. Simultaneous addition of both Cu(II) and Cu(I) also followed the slower $k_{\text{obs II}}$ Cu(I) kinetic trace at 340 nm suggesting mb has a higher affinity for Cu(I) or that the kinetics of binding to Cu(I) as a monomer was faster than
binding to Cu(II) as a dimer. Because of solubility problems with Cu(I) solutions, the thermodynamics of Cu(I) binding by mb were not determined.

As expected Cu(I) did not quench luminescence under anaerobic conditions from either mb or EDTA-mb. The UV-visible absorption spectral changes following Cu(I) additions under anaerobic conditions were identical to those aerobic conditions demonstrating binding. Thus, the oxidation state of copper had no effect on the UV-visible absorption spectra of mb (Figure 8B and C) which was consistent with previous EPR studies (8). Luminescence was quenched in Cu-mb samples following Cu(I) binding under aerobic conditions (Figure 9C). The quenched luminescence by Cu(I) following the addition of air can be explained by the dismutation of aqueous Cu(I) to Cu(II) in the presence of oxygen. When Cu(II) was added to Cu(I)-mb under anaerobic condition, luminescence was also quenched at emission peaks of 310 and 610 nm within 30 s, but not at 461 nm (Figure 9C). The results shown in Figure 9C suggest Cu(I)-mb undergoes a second Cu(II) binding sequence, which would involve Cu(II) coordination to THI and possibly Tyr, followed by reduction to Cu(I) before coordination by HTI. XPS data described above showed mb can reduce more than 2 Cu(II) per mb (Figure 8). Whether HTI remains coordinated to the first Cu(I) through this proposed second binding series and whether Cu(I) remains associated with mb has not been determined.

**Discussion**

The isolation procedure for mb described by Choi et al. (8) provides for the first time mb samples suitable for metal binding studies. Kim et al. (23, 24) have recently
reported the isolation of Cu-s-mb. Samples isolated following copper stabilization are much more resistant to sample breakdown, are less photolabile, and function well in the stimulation of pMMO activity (8, 23). Cu-s-mb also results in a homogeneous sample (8) that probably aided in the crystallization of the molecule for structural characterization (24). However, removal of the copper from Cu-s-mb following exposure to high copper concentrations results in samples with altered spectral (UV-visible absorption, CD, and fluorescence) properties and lower binding constants when compared to mb isolated by the procedures described by Choi et al. (8). The Na₂EDTA treatment described by Kim et al. (23) was successful in the removal of approximately 90% of the copper associated with Cu-s-mb. However, the results presented here suggest the molecule does not return to its original structure. In this study, EDTA-mb provided an interesting comparison to mb, since this sample appears to maintain the Cu-s-mb structure and initially binds Cu(II) and Cu(I) via the THI and HTI groups as predicted from the crystal structure of Cu(I)-mb (23).

With respect to the mechanism of copper binding, the kinetic, spectral, and thermodynamic studies shown here indicate Cu(II) binding was dependant on the Cu(II) to mb ratio and may involve inter- as well as intra-molecular binding similar to that observed with prion proteins (76-78). Formation of distinct Cu-thiolate clusters has also been observed in mammalian metallothioneins at different Cu to metallothionein ratios (51,52). Based on the structure of mb, THI has a longer conjugated system and is probably the group responsible for the absorption maxima at 394 nm. If this prediction is true, our working model for Cu(II) binds involves the following steps (figure 10). Mb initially bound Cu(II) as a dimer (figure 10, I), with Cu(II) coordinated to THI and
possibly Tyr. This proposed initial coordination is based by the lag time and slower kinetic properties when the reaction was monitored at 340 nm. Binding as a dimer was based on the saturation of spectral (UV-visible, CD, and fluorescent) properties and the completed binding energy shift of the thiocarboxyl S state changes at approximately 0.5 Cu(II) per mb. Previous gel filtration chromatography of mb at different Cu(II) to mb ratios also suggesting dimer formation (8). In addition to the THI groups, spectral changes also suggested Tyr was involved in the initial binding of copper by mb. Time course fluorescent changes associated with Tyr following Cu(I) addition were kinetically identical to THI suggesting the quenching of the Tyr signal was coupled with THI (results not shown).

The reduction step (figure 10, II) in the binding process could not be determined directly. However, the absence of quenching of the 461 nm emission following excitation at 340 nm suggests the copper was reduced before coordination to HTI. Emission at 461 nm was quenched in EDTA-mb samples demonstrating emissions from HTI can be quenched if exposed to Cu(II), but not by Cu(I) as shown in the fluorescent spectra of Cu(I) titration under anaerobic conditions. The source of the reductant has not been determined. Like Kim et al. (23), we initially believed the Cys thiols were the source reductants in this reaction. However, the decreased absorption at 254 nm at Cu(II) to mb ratios at or below 0.2 Cu(II) was inconsistent with Cys thiols as the electron source. In addition, attempts to measure Cys thiols chemically or via XPS were unsuccessful. The changes at 254 nm appeared to be more consistent with either a CT between Cu and imidazole nitrogen or to Cys- or thiocarbonyl-S CT. Emission at 461 nm following excitation at 254 nm also suggest absorption at 254 nm is associated with HTI.
The third step (Figure 10, III) in the reaction involves the change in coordination from the thiocarbonyl and N\textsuperscript{\textepsilon} of THI to four thiocarbonyl sulfurs from two THI and two HTI. The proposed change in copper coordination from two THI groups to two THI plus two HTI is based on the lag period between completion of binding at THI and initial coordination to HTI. This change in copper coordination was consistent with previous EPR studies which showed more than one cupric site at low Cu(II) to mb ratios \(8\). XPS data also suggest all four binding sites are through thiocarbonyl sulfur at 0.5 Cu per mb.

The addition of the second Cu(II) to the mb dimer (figure 10, IV \& V) results in the change in coordination from all thiocarbonyl S to a dual N- and S- coordination similar to that shown in the crystal structure of Cu-s-mb \(24\). The change in the Cu(II) binding constant and the increased nitrogen coordination at Cu(II) to mb ratios above 0.5 Cu per mb \(8\) are consistent with this change in copper coordination.

The results presented in this study suggest mb is a dynamic molecule in solution. Monomer, dimer, and potential oligomers of mb have been observed by gel filtration chromatography \(8, 22\). In the absence of copper, mb migrates into several fractions via reverse phase chromatography indicating a mixed population \(8, 23\). Analysis of each fraction by gel filtration or reverse phase chromatography generated chromatographs similar to the original \(8, 22-24\) suggesting mb exists in solution either as a mixture of monomer and oligomers, or the molecule exists in several different conformations. Homogeneous mb samples can be generated via incubation of the samples in the presence of excess concentrations of copper or storage on ice for 2 to 3 days \(8, 23, 24\). These treatments alter the spectral and thermodynamic properties of the sample, but do not alter the stimulatory effects of Cu-mb on methane oxidation by the pMMO \(8\). Whether a
dimer or mixed population theory is used, the results presented here indicate mb is a dynamic molecule in solution and does show properties consistent with a chalkophore.

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References


Table 1. Molar absorption coefficients (ε) of mb, and Cu-mb.

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<th>Metal</th>
<th>Wavelengths (nm)</th>
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<td>-</td>
<td>394 - 422</td>
<td>7.14</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>340 - 422</td>
<td>8.1</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Cu(II)</td>
<td>394 - 422</td>
<td>-</td>
<td>2.39</td>
<td>4.75</td>
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<tr>
<td>Cu(II)</td>
<td>340 - 422</td>
<td>5.16</td>
<td>2.92</td>
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Table 2. Thermodynamic parameters for Cu(II) binding to mb at pH 6.8. Monomer and dimer model was based on best fits using one or two binding site model.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>mb</th>
<th>mb&lt;sub&gt;2&lt;/sub&gt;</th>
<th>mb&lt;sub&gt;2&lt;/sub&gt; EDTA Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Monomer Model</td>
<td>Dimer Model</td>
<td>Dimer Model</td>
</tr>
<tr>
<td>N&lt;sub&gt;1&lt;/sub&gt; (Cu mb&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.495 ± 0.005</td>
<td>0.11 ± 0.002</td>
<td>-</td>
</tr>
<tr>
<td>K&lt;sub&gt;1&lt;/sub&gt; (M&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>1.12 ± 0.16 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>&gt; 8 x 10&lt;sup&gt;18&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>ΔH&lt;sub&gt;1&lt;/sub&gt; (kcal mol&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>-29.8 ± 0.41</td>
<td>~ -146</td>
<td>-</td>
</tr>
<tr>
<td>ΔS&lt;sub&gt;1&lt;/sub&gt; (cal mol&lt;sup&gt;-1&lt;/sup&gt; deg&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>-72.2</td>
<td>~ -331</td>
<td>-</td>
</tr>
<tr>
<td>ΔG&lt;sub&gt;1&lt;/sub&gt; (kcal mol&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>-8.25</td>
<td>~ -47</td>
<td>-</td>
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<tr>
<td>N&lt;sub&gt;2&lt;/sub&gt; (Cu mb&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>-</td>
<td>0.14 ± 0.013</td>
<td>0.14 ± 0.003</td>
</tr>
<tr>
<td>K&lt;sub&gt;2&lt;/sub&gt; (M&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>-</td>
<td>2.6 ± 0.47 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>2.3 ± 0.41 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
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<tr>
<td>ΔH&lt;sub&gt;2&lt;/sub&gt; (kcal mol&lt;sup&gt;-1&lt;/sup&gt;)</td>
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<td>-29.3 ± 0.38</td>
<td>-</td>
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<tr>
<td>ΔS&lt;sub&gt;2&lt;/sub&gt; (cal mol&lt;sup&gt;-1&lt;/sup&gt; deg&lt;sup&gt;-1&lt;/sup&gt;)</td>
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<td>-11.46</td>
<td>-9.7</td>
<td>-</td>
</tr>
<tr>
<td>N&lt;sub&gt;3&lt;/sub&gt; (Cu mb&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>-</td>
<td>0.37 ± 0.02</td>
<td>0.27 ± 0.017</td>
</tr>
<tr>
<td>K&lt;sub&gt;3&lt;/sub&gt; (M&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>-</td>
<td>1.40 ± 0.21 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>6.7 ± 0.11 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
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<tr>
<td>ΔH&lt;sub&gt;3&lt;/sub&gt; (kcal mol&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>-12.83 ± 0.49</td>
<td>-11.4 ± 0.79</td>
<td>-</td>
</tr>
<tr>
<td>ΔS&lt;sub&gt;3&lt;/sub&gt; (cal mol&lt;sup&gt;-1&lt;/sup&gt; deg&lt;sup&gt;-1&lt;/sup&gt;)</td>
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<td>6.47 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2.78 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Figure 1. Schematic drawing of copper-containing methanobactin modified from Kim et al. (24). Abbreviations: THI, 4-thiocarbonyl-5-hydroxylimidazolate; HTI, 4-hydroxy-5-thiocarbonylimidazolate.
Figure 2. (A) UV-visible absorption spectra of mb isolated with a Cu to mb molar ratio of 0.01 Cu per mb and following the addition of 0.003, 0.015, 0.03, 0.06, 0.1, 0.2, 0.3, 0.4, 0.6, and 1.0 Cu(II) atoms per mb. (B and C) Absorption changes at 394 nm following Cu(II) additions. (D) Absorption changes at 254 (●), 282 (□), 302 (▲), and 340 nm (○) following Cu(II) additions. Scale on right was used for absorption changes at 340 nm and scale on left was used for absorption changes at 254, 282, and 302 nm.
Figure 3. Kinetics of Cu(II) binding by mb. Absorption changes at 340 (■) and 394 nm (▲) following the addition of 3 Cu(II) per mb at 20°C. Arrow indicates the time when the mixing flow stopped.

Figure 4. (A) Emission spectra of mb in aqueous solution with different excitation wavelength (nm), $\lambda_{ex} = 282$ nm, 340 nm, and 394 nm at ambient temperature (thick lines). Arrows indicate the direction of spectrum changes upon copper addition and thin lines show the spectra upon completion of changes. (B - D) Percent emission spectrum changes to mb (▲, ■, and ●) and EDTA-mb (△, □, and ○), emission spectrum changes were monitored at (B) 310 nm ($\lambda_{ex} = 280$ nm), at (C) 461 nm ($\lambda_{ex} = 340$ nm), and at (D) 610 nm ($\lambda_{ex} = 394$ nm).
Figure 5. Circular dichroism spectra of mb (A & C) and EDTA-mb (B) as isolated (thick line) and following addition 0.1 to 1.0 molar equivalents of Cu(II) (thin lines) (A & B) or the addition of 0.1 to 2.0 molar equivalents of Cu(I) (thin lines) (C). Panels D - F illustrate the effect of Cu(II) (D & E) or Cu(I) (F) on the CD at 201 (○), 216 (◇), 317 (▲), and 410 (■) nm.

Figure 6. A. Oxidation states of copper at different copper to mb molar ratios as determined by X-ray photoelectric spectroscopy. B. Sulfur XPS spectra of mb (a) and following the addition of 0.25 (b), 0.5 (c) and 0.75 (d) molar equivalents of Cu(II).
Figure 7. Thermogram (A) and binding isotherm (B) showing additions of CuSO\textsubscript{4} (60 separate 5 µl injections from 800 µM CuSO\textsubscript{4} solution) into 100 µM mb (cell) in water at 25°C. Binding isotherm showing the curve fitting for a one site-binding algorithm (C), and binding isotherm showing the curve fitting for a two site-binding algorithm (D). E and F, binding isotherm showing the curve fitting for a two site-binding algorithm at low Cu(II) concentrations, copper and mb concentrations in E and F were raised to 1.6 mM and 800 µM, respectively.
Figure 8. (A) UV-visible absorption spectra of mb following the addition of 0 to 2.0 Cu(I) atoms per mb in 0.1 molar increments. (B) Absorption changes at 254 (○), 282 (□), 302 (▲), 340 nm (○), and 394 nm (●) following Cu(I) additions. Scale on the left was used for absorption changes at 282, 340 and 394 nm and scale on the right was used for absorption changes at 254 and 302 nm.
Figure 9. (A) UV-visible absorption spectra of mb from *Ms. trichosporium* OB3b spectra taken every 30 s following the addition of CuCl. (B) Percent absorption change over time at 340 (○) and 394 (△), and at 340 (●) and 394 (▲) in the presence of Cu(II), arrow marks time of Cu(II) addition. Insert, solubilization of CuCl by mb, A, H2O blank; B, 100 µM CuCl to 100 µM mb; C, 100 µM mb; D, 6 min after addition of 100 µM CuCl to 100 µM mb. (C) UV-visible absorption changes at 340 nm (○) and 394 nm (△), and emission intensity changes at 310 nm (λex = 280 nm) (●), 461 nm (λex = 340 nm) (■), and 610 nm (λex = 394 nm) (□) following addition of 3 molar excess of CuCl under anaerobic conditions. Arrow indicates time of exposure to air (O2).
Figure 10. Model for Cu(II) binding by mb. Abbreviations/symbols; Yellow symbol, thiocarbonyl group; blue symbol, imidazole $N^\varepsilon$ atom; HTI, 4-hydroxy-5-thiocarbonyl imidazolate; THI, 4-thiocarbonyl-5-hydroxy imidazolate.
CHAPTER 5: SPECTRAL AND THERMODYNAMIC PROPERTIES OF Ag(I), Au(III), Cd(II), Fe(III), Hg(II), Mn(II), Ni(II), Pb(II), U(IV), and Zn(II) BINDING BY METHANOBACTIN FROM Methylosinus trichosporium OB3b

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Abstract

Methanobactin (mb) is a novel chromopeptide that appears to function as the extracellular component of a copper acquisition system in methanotrophic bacteria. To examine this potential physiological role, and to distinguish it from iron binding siderophores, the spectral (UV-visible absorption, circular dichroism, fluorescence, and X-ray photoelectron) and thermodynamic properties of metal binding by mb were examined. In the absence of Cu(II) or Cu(I), mb will bind Ag(I), Au(III), Co(II), Cd(II),

Fe(III), Hg(II), Mn(II), Ni(II), Pb(II), U(VI), or Zn(II), but not Ba(II), Ca(II), La(II), Mg(II), and Sr(II). The results suggest metals such as Ag(I), Au(III), Hg(II), Pb(II) and possibly U(VI) are bound by a mechanism similar to Cu, whereas the coordination of Co(II), Cd(II), Fe(III), Mn(II), Ni(II) and Zn(II) by mb differs from Cu. Consistent with its role as a copper-binding compound or chalkophore, the binding constants of all the metals examined were less than those observed with Cu(II) and copper displaced other metals except Ag(I) and Au(III) bound to mb. However, the binding of different metals by mb suggests that methanotrophic activity also may play a role in either the solubilization or immobilization of many metals in situ.

**Introduction**

Methanobactin (mb) is a low molecular mass (1,154 Da) chromopeptide observed in both the extracellular and membrane fraction in many if not all aerobic methanotrophs [1-5]. When isolated from the membrane fraction, mb contains one copper atom and is predominately associated with the membrane-associated or particulate methane monooxygenase [5-7]. In the extracellular fraction, the majority of mb is metal free [2, 5], and appears to be the extracellular component of a copper acquisition system similar to bacterial siderophore-based iron acquisition systems [2-6, 8-12]. This proposed copper-siderophore, or chalkophore role [3], is based on copper uptake and localization studies [2, 4-7, 11], chelation of copper in soil systems [11], characterization of constitutive soluble methane monooxygenase mutants in *Ms. trichosporium* OB3b [2, 4, 9, 12], and copper-binding studies [2, 5, 6, 8, 10].

The structure of copper containing mb (Cu-mb) following exposure to high
copper concentrations showed the molecule bound one copper atom in a novel S, and N coordination by the 4-thiocarbonyl-5-hydroxy imidazolate (THI) and 4-hydroxy-5-thiocarbonyl imidazolate (HTI) moieties [3]. However, spectral, kinetic and thermodynamic studies indicate that initial coordination of Cu(II) and Cu(I) differs from the coordination observed in the crystal structure [8]. Mb appears to initially coordinate Cu(II) as tetramer or oligomer by THI and possibly Tyr (Fig. 1). This initial coordination is followed by a reduction of Cu(II) to Cu(I), and then followed by a change in metal ligation resulting in coordination by both THI and the HTI. At Cu(II) to mb ratios above 0.25 the Cu(II) is coordinated as a dimer, followed by coordination as a monomer at Cu(II) to mb ratios above 0.5 Cu per mb (Fig. 1).

The structural similarities of mb to siderophores in the pyoverdin class [13-16] suggested that mb may prove to be a siderophore with a capacity to bind Cu(II) as well as Fe(III). Several other observations suggest mb may be involved in the mobilization of non-cuprous metals. The coupled increase in iron uptake with increased copper uptake, or copper-induced iron uptake, suggest that mb may be involved in iron uptake [5, 17]. Given that mb is the major if not sole extracellular metal binding compound produced by Ms. trichosporium OB3b [2, 6, 8, 10], the observation by Jenkins et al. [18] that this bacterium mobilizes Cd(II) in soil columns suggest mb may bind Cd(II). To determine if mb can function as a siderophore and/or to mobilize metals other than copper, the metal binding properties of mb were examined. In this report the initial spectral and thermodynamic properties of Ag(I), Au(III), Co(II), Cd(II), Fe(III), Hg(II), Mn(II), Ni(II), Pb(II), U(VI) and Zn(II) binding were examined with special attention given to metals which are coordinated and reduced via a mechanism similar to copper, i.e. Ag(I),
Au(III), Hg(II), and Pb(II). The results show that mb is primarily involved copper mobilization, but the binding of different metals by mb suggests that methanotrophic activity also may play a role in solubilization of many metals in situ.

**Experimental**

**Organisms culture conditions and isolation of mb.**

*Ms. trichosporium* OB3b was cultured in either 0 or 0.2 µM CuSO₄ amended nitrate minimal salts (NMS) medium as previously described [6]. Mb was prepared from *Ms. trichosporium* OB3b as described previously [6, 8] the rotary evaporation treatment was removed from the isolation procedure. Instead the methanol was removed during lyophilization. For preparations of metal saturated mb samples, CdCl₂, CoCl₂, FeCl₃, MgCl₂, MnCl₂, NiCl₂, or ZnSO₄ was added to the spent media to a final concentration of 2 mM followed by 8 h incubation in the dark at 4°C. The spent medium was then centrifuged twice at 15,000 x g for 20 min to remove metal precipitations and loaded on a 7 x 20 cm Dianion HP-20 column (Supelco, Bellefonte, PA). Bound metal-mb were washed with 4 column volumes of H₂O and eluted with 60% methanol:40% H₂O and lyophilized. Due to precipitation or altered column binding properties following exposure to excess metal concentrations, the concentration of Au(III), Fe(III), Hg(II), and U(IV) bound by mb were determined via titration experiments (see below)

**Metal titrations.**

Metal titration experiments were determined by addition of 100 µM, 1 mM, or 10 mM solutions of AgNO₃, HAuCl₄, CdCl₂, CoCl₂, CrO₃, CuSO₄, FeCl₃, HgCl₂, MgCl₂,
MnCl₂, NiCl₂, Pb(NO₃)₂, UO₂(NO₃)₂, or ZnSO₄ to 50 µM mb dissolved in H₂O, pH 6.8 as previously described for Cu(II) or Cu(I) titrations [8] unless otherwise stated. Glassware was soaked in 0.1 N HNO₃ for 12 h then rinsed with milliQ H₂O. For the metal replacement experiments, 50 µM aqueous mb solutions were preloaded with equimolar of AgNO₃, HAuCl₄, CaCl₂, CdCl₂, CoCl₂, CrO₃, CuSO₄, FeCl₃, HgCl₂, MgCl₂, MnCl₂, NiCl₂, Pb(NO₃)₂, UO₂(NO₃)₂, or ZnSO₄, incubated for a 10 min followed by the addition of equimolar CuSO₄ then monitored via UV-visible absorption spectroscopy every 30 s for 0.5 to 120 min. Between scans the samples were stored in the dark to avoid photodegradation [6].

**Spectroscopy, isothermal titration calorimetry (ITC), and metal determinations.**

UV-visible absorption spectra, fluorescence spectra, and metal determinations via inductively coupled plasma atomic emission-mass spectroscopy (ICP-MS) were determined as previously described [7, 8]. In contrast to a previous report [8], the baseline was used as a reference point instead of the isosbestic points in UV-visible absorption spectra for the comparison of Δεs.

CD spectra measurements were carried out on either a JASCO J-710 spectropolarimeter (Jasco Co, Tokyo, Japan) or on a Applied Photophysics SX.18MV CD spectrophotometer as previously described [8]. Metals were titrated into 100µM aqueous mb solution.

EPR samples were prepared by adding equimolar metals to 5mM mb aqueous solutions. After 5 min of incubation, samples were transferred to quartz EPR tubes, then frozen in a liquid nitrogen bath, and the spectra determined as previously described [8].
ITC was performed with following modifications from the previously described procedure [8]. First, concentrations of the titrant and cell solutions were raised to 3.2mM and 0.4mM, respectively. Second, the interval between titrant injections were decreased to 600 s and the stirring rate decreased to 380 rpm.

X-ray photoelectron spectroscopy (XPS).

XPS was performed on a model Phoibos-150 hemispherical analyzer (SPECS Scientific Instruments, Sarasota, FL) or on a model 5600ci spectrophotometer (Perkin-Elmer Inc., Eden Prairie, MN) as previously described [8].

Transmission electron microscroscopy.

Gold nanoparticle production was determined by addition of 10 mM aqueous solutions of HAuCl₄ to 1 or 5 mM aqueous mb solutions. Mb solutions were prepared freshly and immediately dispensed into 1.8 ml glass vials. Gold solutions were added to the glass vials containing mb solutions to with a final molar ratios of 0, 0.1, 0.2, 0.4, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 10.0, 15.0, or 20.0 Au(III) to mb. All samples were incubated for 15 min with or without stirring. In some cases the Au-mb solutions were spotted on formvar coated Ni or Cu grids. Other samples were subjected to one freeze thaw cycle before spotting on formvar coated Ni or Cu grids. Lastly some samples were centrifuged at 10,000 x g for 2 min at room temperature and the loose red pellet spotted on formvar coated Ni grids. The samples on Ni or Cu grids were then dried under vacuum and examined with a JEOL 1200X scanning/transmission electron microscope.
Results and discussion

Metals bound by mb and metals binding groups.

Initial screening by UV-visible absorption spectroscopy showed that in the absence of Cu(II) or Cu(I), mb will bind Ag(I), Au(III), Cd(II), Co(II), Fe(III), Hg(II), Mn(II), Ni(II), Pb(II), U(VI), or Zn(II) but not Ba(II), Cr(VI), La(III), Mg(II), or Sr(II) (see below). Based on the redox and spectral properties described below, the metals bound by mb were placed into two groups. Choi et al. [8] recently described the spectral and thermodynamic properties of Cu(II) and Cu(I) which are designated in mb group A metals. Metals that showed a final coordination via 4-thiocarbonyl-5-hydroxy imidazolate (THI), 4-hydroxy-5-thiocarbonyl imidazolate (HTI) and possibly Tyr were placed in group A. In general, mb also reduced these metals without the addition of an external reductant. Lastly, depending on the metal concentration, mb coordinated group A metals as either a tetramer, dimer, or monomer. In addition to Cu, Group A metals include Ag(I) and Au(III), Hg(II), Pb(II) and possibly U(VI).

Group B metals consist of the transition metals Cd(II), Co(II) Fe(III), Mn(II), Ni(II), and Zn(II). Group B metals were characterized by a final coordination to THI and without a change in the oxidation state of the metal. In addition, depending on the concentration of group B metal, mb coordinated group B metals as either a tetramer or dimer, but not as a monomer. Thus, even in the presence of excess metals, mb coordinates group B metals as a dimer. With respect to the copper binding model proposed by Choi et al. [8], coordination of group B metals stops after the initial two binding step (Fig. 1).
**UV-visible absorption spectra.**

**Group A metals.** As observed with copper [8], the binding of Au(III) (Figs. 2A & 2B), Ag(I) (Figs. 3A & 3B) and U(VI) (results not shown) resulted in a decreased absorption at 394 nm suggesting coordination to THI (Table 1). In addition to a decrease in absorption at 394 nm, the addition of Hg(II) or Pb(II) also resulted in a shift in the absorption maxima to 385 and 400 nm, respectively (results not shown). Spectral changes were also observed at 340 nm following the addition of group A suggesting coordination to HTI. However, in contrast to Cu(I), Cu(II) and U(VI) which showed a decreased absorbance at 340 nm following copper binding [8], the spectral changes associated with HTI following the addition of other group A metals were complex. At Au(III) or Hg(II) concentrations \( \leq 0.3 \) metal per mb, a red shift in the absorption maximum from 340 to 363 nm was observed with an increased absorption at 363 nm (Figs. 2A and 2B). At Au(III) or Hg(II) concentrations > 0.3 metal per mb, a decrease in absorbance at 363 nm was observed. A similar response to metal concentration was observed at 302 nm, where an increase in absorbance occurred at low metal concentrations (i.e. \( \leq 0.3 \) metal per mb) followed by a decreased absorbance at metal to mb ratios between 0.3 and 1.0 metal per mb and an increased absorbance at metal to mb concentrations above 1.0. The spectral changes associated with Hg(II) were identical to Au(III) (results not shown).

The spectral changes at 302 nm following the addition of Ag(I) were identical to Au(II) with an initial increase a Ag(I) to mb ratios \( \leq 0.3 \) Ag(I) per mb, followed by a decrease at higher Ag(I) to mb ratios. The spectral shifts at 340 nm following the addition of Ag(I) were also similar to Au(II) and Hg(II) with a red shift to 367 nm, however, the
changes in absorption were opposite to that observed with Au(II), with an initial decrease in absorption from HTI nm at Au(I) concentrations ≤ 0.3 Au(I) per mb followed by an increased absorption at higher Ag(I) concentration (Figs. 3A and 3B). The spectral changes associated with HTI following the addition of Pb(II) resulted in a decrease in absorption along with a spectral shift to 350 nm (results not shown). The results suggest coordination to HTI for all group A metals, but the coordination may differ between members of the group A metals and that the interactions between metal and HTI may change at different metal to mb ratios.

**Group B metals.** The binding of group B metals by mb resulted in a decreased absorption at 394 nm, with either no change (Ni and Fe), or an increased in absorption at 340 nm (Cd, Co, and Zn) (Fig. 4; Table 1). In the case of Mn(II), a blue shift in the maxima of at 394 nm to 377 nm following metal addition was also observed (results not shown). In addition, with the exception of Ni(II) and Mn(II), little to no changes in absorbance were observed in the 250 – 310 nm range for this metal group. Mn(II) addition resulted in an decrease in absorption at 302 nm without an associated increase in absorption at 282 nm. The absence of an absorbance change at 282 nm with decreased absorption at 302 nm suggests the absorption maxima at 282 and 302 nm do not represent the phenolic and phenoxide ion forms of Tyr and may represent a charge transfer band [8, 19].

The final spectral changes associated with the binding of group B metals were similar to those observed in the initial coordination to Cu(II) suggesting these metals were bound as a dimer via the THI moieties (Fig. 1) [8]. To determine if the final coordination of group B metals was a dimer, the concentration of metal associated with
mb were determined following exposure to excess metal concentrations. Consistent with
the UV-visible absorption titrations, the concentration of Cd(II), Co(II), Mn(II), Ni(II), or
Zn(II) bound to mb was approximately half of that observed with Cu(II) demonstrating
mb binds these metals as a dimer even in the presses of saturating metal concentrations
(Table 2).

Fluorescence Spectroscopy.

As observed with Cu, [8], the addition of other group A, and with the exception of
Mn(II), group B metals quenched emissions from THI following excitation at 394 nm
($\lambda_{ex394}$) (Figs. 2E, 2F, 3E, and 3F; Table 3). The addition of Mn(II) had no effect on
emission from THI (results not shown). With the exception of Co(II) and Mn(II), the
addition of group A and B metals also quenched emission from Tyr suggesting Tyr was
either involved in metal coordination or was proximal to the metal coordination site
(Figs. 2E, 2F, 3E, and 3F; Table 3).

The addition of groups A and B metals had mixed effects on emission from HTI
following excitation at 340 nm (Figures 3E and 3F). The addition of Cd(II), Zn(II),
Hg(II) and Au(III) to mb resulted in an increase in emission from HTI (Figures 4E, and
4F) following excitation at 254, 340, or 394 nm. In the case of Au, the emissions
observed at 461 nm decreased at Au(III) to mb ratios $\geq 0.8$ following excitation at 254
and 340 nm with new emission maxima at 421, 441, and 524 nm following excitation at
394 nm (Figs. 5A and 5B). These new emission maxima were not observed with
excitation at 254 or 340 nm nor were they observed with Hg(III). Cation induced
fluorescence has been shown to occur with removal or separation of an internal quencher
following cation binding, or via cation binding to the internal quencher [20, 21]. Cation induced increased fluorescence has also been observed in chlorophyll \(a\) in the presence of negatively charged gold nanoparticles [22]. In this analogy gold nanoparticles functioned as an electron shuttle from an electron source such as THI to HTI. XPS spectroscopy demonstrated the reduction of Au(III) to Au(0) and examination of Au-mb complexes by transmission electron microscopy (TEM) and UV-visible absorption spectroscopy showed the formation of nanoparticles at Au to mb ratios above 1.2 Au per mb (see below).

**Circular Dichroism Spectroscopy.**

The UV-CD spectrum of mb showed a strong negative band below 200 nm with negative shoulders at 202 nm and 217 nm (Figures 2C and 4C), characteristic of an unordered polypeptide [23]. Like the UV absorption spectra, the CD spectra of the group A metals were complex and depended on the metal to mb ratio (Fig 2). At Au(III) (Figure 2C and 2D) or Hg(II) to mb concentrations \(\leq 0.4\) metal to mb, the UV-CD spectra was similar to that of Cu [8]. However, at concentrations of metal to mb \(> 0.5\), the trend reversed. The UV-CD spectra of Ag(I) was also complex with the development of an absorption maxima at 288 nm at Ag(I) to mb ratios \(\leq 0.7\) Ag(I) per mb followed by a red shift to 298 nm at Ag(I) to mb ratios between 0.7 and 0.9 Ag(I) per mb (Fig. 3C and 3D). At Ag(I) to mb ratios \(\geq 0.9\) nm, the absorption intensity at 298 nm does not change, but a new absorption maxima at 318 nm was observed. The absorption maxima between 286 and 318 nm may represent both changes in both the environment and oxidation state of the Tyr. Addition of group B resulted in positive band enhancements at 190 nm,
suggesting the development of α-helical characteristics (Fig. 4C and 4D).

The visible CD spectra following metal binding showed the development of an exciton coupled spectrum between the two-chromophore system (THI and HTI) with all metals tested (Figs 2-4). The CD spectra in the 315 to 415 nm region following metal additions were consistent with a Cotton effect involving the THI and HTI [23-25]. At molar ratios < 0.3 Au(III) or Hg(II) per mb, the visible-CD spectra were similar to that observed following copper addition (Figs. 2C, 2D) [8]. The CD-spectra associated with HTI following the addition of Au(III) or Ag(I) resulted in a red shift from 340 nm to 360 and 354 nm, respectively, which were similar to that observed in the UV-visible absorption spectra, with an associated negative band enhancement. The CD-spectra from THI showed a positive band enhancement with little or no shift in the absorption maxima. The absence of a shift in the absorption maxima of both THI and HTI in the CD-spectra suggest little to no change in the hydrophobicity of the environment of these groups following metal binding. In contrast to Cu [8], little change in the visible CD spectra was observed at Au(III) or Hg(II) to mb ratios between 0.3 and 0.5 (Figs. 2C and 2D) and between 0.3 and 0.6 Ag(I) per mb (Figs. 3C and 3D) suggesting little to no changes in the orientation between THI and HTI occurred in this concentration range. At Au(III) or Hg(II) concentrations >0.5 per mb, the trends throughout the visible CD-spectra reversed and with the exception of the spectral shift of HTI, the spectra at 2.0 Au(II) or Hg(II) per mb were similar to metal free mb. At Ag(I) to mb ratios > 0.6, the spectral changes were essentially opposite to that observed with Au(III) or Hg(II) (Fig. 3C and 3D).

In group B a negative band enhancement near 340 nm (2nd Cotton effect, HTI) and a positive band enhancement between 360 nm and 370 nm (1st Cotton effect, THI)
were observed with metal addition suggesting the two chromophores were brought together with a counter-clockwise twist (positive chirality) (Fig. 4C and 4D) [23-25]. In contrast to Cu [8], the absorbance maxima associated with HTI following the addition of group B metals remained near 340 nm, suggesting the hydrophobicity of the environment around HTI did not change following metal binding. The absorption maxima associated with THI showed a blue shift indicating THI moved to a more hydrophobic environment following the binding of groups B metals. This spectral shift was opposite to the at observed with Cu suggesting the conformation changes associated with the binding of groups B metals were in an opposite rotation to the changes associated with the coordination of Cu(II) or Cu(I) [8].

In contrast to copper [8], no strong relationships between Tyr and HTI were observed in the CD spectra following the addition of other group A and groups B metals (Figs 2 – 4).

Electron paramagnetic resonance (EPR) and X-ray photoelectron spectroscopy (XPS): oxidation state of metals bound to mb.

X-band EPR spectra of Fe-mb, Co-mb, and Mn-mb samples suggest metal coordination, but not reduction by mb (Fig. 6). Ferric saturated mb samples showed a narrowing of the $g = 4.3$ peak suggesting coordination and possible cluster formation similar to that observed with a variety of siderophores (Fig. 6A) [26]. XPS-spectroscopy of Fe-mb complexes confirmed iron associated with mb remained in the ferric state, in contrast to Cu(II) which is reduced to Cu(I) [2, 3, 5, 8]. The EPR spectra of Co-mb was narrower than Co(II), again suggesting coordination without reduction (Fig. 6B). Ni-mb
showed no Ni EPR signal, which was surprising considering XPS spectra showed the oxidation state Ni(II) did not change following binding to mb.

XPS showed that the group A metal, Au(III), was reduced to Au(0) by mb. Like Cu(II) more than one Au(III) were reduced per mb [8] (Fig. 7). In fact, Au(III) was not detected in reaction mixtures until the Au to mb ratio was > 2 Au(III) per mb. Examination of Au-mb complexes by transmission electron microscopy (TEM) showed the Au(0) remained associated with mb even at high Au(0) to mb ratios with little to no detection of nanoparticles (Figs. 8A – 8C). However, if samples were centrifuged or subjected to one freeze thaw cycle nanoparticle formation was observed at Au to mb ratios above 1.2 Au per mb (Figure 9D). Following a freeze-thaw cycle or centrifugation, the nanoparticle sizes ranged from 2.5 to 30 nm, with the majority (60%) in the 11 – 20 nm particle range. If Au-mb solutions were examined on formvar coated copper grids nanoparticle formation was also observed (Figure 8 E and 8F). Nanoparticles formed on copper grids were significantly smaller, average particle size 3.7 ± 1.1 nm, than following centrifugation or a freeze-thaw cycle. The oxidation states of Hg and Ag bound to mb were not determined, but formation of insoluble gray to black precipitates following the addition of Hg(II) or Ag(I) suggested these metals were also reduced by mb.

Metal free mb shows two sulfur signals, one at approximately 163.3 eV which has been assigned to Cys and Met S [8, 27-29] and one at 161.4 eV [8, 27-29] which has been attributed to thiocarbonyl S [8] (Fig. 9). As with the addition of Cu(II) [8], the addition of Au(III) resulted in an increased signal intensity at 163.3 eV and a binding energy shift of the thiocarbonyl S at 161.3 eV. The increased signal intensity at 163.3 eV and the binding energy shift of the thiocarbonyl S were complete at Au:mb ratios ≤ 0.3 Au per
mb suggesting that mb initially binds Au as a tetramer. The similarity in the concentration of Cu(II) [8] and Au(III) required to complete the binding energy shift of the thiocarbonyl S was unexpected since Au(III) binding is followed by a three electron reduction and Cu(II) binding results in a one electron reduction suggesting the energy shift in the thiocarbonyl S followed metal binding and not necessary a change in oxidation state. A previous publication [8] reported the binding energy shift of the thiocarbonyl S occurred at Cu(II) to mb ratios ≤ 0.5. However, a more complete titration with Cu(II) has shown the binding energy shift of the thiocarbonyl S was complete at Cu(II) to mb ratios ≤ 0.3 Cu(II) per mb (results not shown).

**Isothermal titration calorimetry (ITC).**

With the exception of Co(II), Pb(II) and Mn(II), all of the metals examined fit a two-site binding model better than a one-site binding model (Table 4). Most of the metals followed a titration curves similar to Hg(II) (Fig. 10A), Ni(II) (Fig. 10D) or Ag(I) (Fig. 10C), with extreme transitions observed with Au(III) (Fig. 10B) and Zn(II) (Fig 10E). The cause for the initial increase in free energy change with increased Au(III) concentration was not determined, but may be associated with the formation of Au(0) nanoparticles (Figure 10B). The reason for the a transition from exothermic to endothermic in Zn(II) titrations was not determined (Fig. 10E). The binding constants observed with non-Cu group A and group B metals were well below the binding constants observed with Cu(II) (Table 4, Fig. 10) and is consistent with its proposed role as a chalkophore [8].
**Summary and concluding remarks**

In contrast to iron siderophores, which are generally specific for Fe(III) [14, 16, 30-32], the results presented here show mb will bind a variety of metals. The binding of different metals by mb is intriguing and suggests that although mb preferentially binds copper, mb produced by methanotrophs may play a role in solubilization of many metals in situ. One of the persistent and substantial problems in remediation of hazardous waste sites is the mobilization and transport of radionuclides and heavy metals from these sites to surrounding areas [33-39]. Methanotrophic bacteria are often present at these sites and often used in the remediation of halogenated hydrocarbons [40]. The results presented in this report indicate they may also be responsible or involved in the mobilization of radionuclides and heavy metals. For example, studies by Jenkins et al. [18] showed that soluble extracellular extracts produced by methanotrophs increased the transport of Cd(II) in porous soil columns. On the other hand, the reduction of several group A metals can also result in the metal immobilization.

The mechanism of metal binding by non-Cu group A metals showed a number of similarities originally observed with Cu group. First, at low metal concentrations, mb appeared to bind non-Cu Group A metals as a tetramer or oligomer via THI and HTI. Second, all of Group A metals tested were reduced by mb. Third, at metal to mb ratios between 0.25 and 0.5 metal per mb, the metals are coordinated via a mb dimer followed by a monomer at equimolar metal to mb concentrations. Fourth, at least in the case of Au, more than one metal atom was reduced per mb. Taken together the results suggest non-Cu group A metals followed a metal binding and reduction scheme similar to copper for all group A metals (Fig. 1). However, the CD-spectra suggest the final conformation
changes associated with non-Cu group A metals differed from that observed with following copper binding.

The results presented here also suggest the mechanism of binding to groups B metals differs from that observed with group A metals [8]. Mb appears to bind group B metals as a tetramer or dimer depending on the metal concentration via THI (Fig. 1). With respect to the mechanism of binding, group B metals appear to follow the initial binding steps observed with group A metals which also initially binds copper via THI (Fig. 1) [8].

**Abbreviations**

\(\Delta A\), absorption change;

CD, circular dichroism;

CT, charge transfer;

Ag-mb, methanobactin silver complex;

Ag-mb, methanobactin gold complex;

Cd-mb, methanobactin cadmium complex;

Co-mb, methanobactin cobalt complex;

Cu-mb, methanobactin copper complex;

Fe-mb, methanobactin iron complex;

Hg-mb, methanobactin mercury complex;

Mn-mb, methanobactin manganese complex;

Ni-mb, methanobactin nickel complex;

Pb-mb, methanobactin lead complex;
U-mb, methanobactin uranium complex;
Zn-mb, methanobactin zinc complex;
EPR, electron paramagnetic resonance;
HTI, 4-hydroxy-5-thiocarbonyl imidazolate;
ITC, isothermal titration calorimetry;
K, binding constant;
Mb, methanobactin;
MMO, methane monooxygenase;
pMMO, membrane-associated methane monooxygenase;
sMMO, soluble methane monooxygenase;
THI, 4-thiocarbonyl-5-hydroxy imidazolate;
TEM, transmission electron microscopy;
XPS, X-ray photoelectron spectroscopy

**Acknowledgements**

We thank V. Frasca at Microcal for assistance in modeling of the ITC results and Tracey M. Pepper (ISU Microscopy Facility) for TEM analysis. This work was supported by the Department of Energy grant 02-96ER20237 (to AAD & WEA), Department of Energy grant DE-FC26-05NT42431 (to JDS), an Inland Northwest research Alliance Graduate Fellowship grant to ESB, National Science Foundation Career grant MCB 0349139 and Cottrell awards (NLP), and the Plant Sciences Institute and Department of Biochemistry, Biophysics, and Molecular Biology for assistance in purchasing the ITC.
References


Table 1. Molar absorption coefficients ($\varepsilon$) of mb and metal-mb.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>$\varepsilon_{340}(\text{mM}^{-1}\text{cm}^{-1})$</th>
<th>$\Delta\varepsilon_{340}(\text{mM}^{-1}\text{cm}^{-1})$</th>
<th>$\varepsilon_{394}(\text{mM}^{-1}\text{cm}^{-1})$</th>
<th>$\Delta\varepsilon_{394}(\text{mM}^{-1}\text{cm}^{-1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>mb</td>
<td>18.24</td>
<td>-</td>
<td>16.07</td>
<td>-</td>
</tr>
<tr>
<td><strong>Group A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cu-mb</td>
<td>13.55</td>
<td>4.69</td>
<td>9.75</td>
<td>6.31</td>
</tr>
<tr>
<td>Au-mb</td>
<td>9.01</td>
<td>9.23</td>
<td>7.07</td>
<td>9.00</td>
</tr>
<tr>
<td>Hg-mb</td>
<td>11.57</td>
<td>6.67</td>
<td>12.92</td>
<td>3.14</td>
</tr>
<tr>
<td>U-mb</td>
<td>16.24</td>
<td>2.00</td>
<td>13.98</td>
<td>2.09</td>
</tr>
<tr>
<td><strong>Group B</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cd-mb</td>
<td>18.73</td>
<td>-0.49</td>
<td>11.33</td>
<td>4.74</td>
</tr>
<tr>
<td>Co-mb</td>
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<td>12.78</td>
<td>3.29</td>
</tr>
<tr>
<td>Fe-mb</td>
<td>18.17</td>
<td>0.07</td>
<td>10.19</td>
<td>5.88</td>
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<tr>
<td>Mn-mb</td>
<td>20.54</td>
<td>-2.30</td>
<td>11.28</td>
<td>4.79</td>
</tr>
<tr>
<td>Ni-mb</td>
<td>17.88</td>
<td>0.36</td>
<td>12.21</td>
<td>3.86</td>
</tr>
<tr>
<td>Zn-mb</td>
<td>19.69</td>
<td>-1.45</td>
<td>11.13</td>
<td>4.94</td>
</tr>
</tbody>
</table>

*An increase in absorbance was observed.
Table 2: Molar ratios of Cu(II) and group B metals per mb. Mb was treated with an saturating concentrations of (50 fold molar excess) of Cu(II) (Cu-mb), Cd(II) (Cd-mb), Zn(II) (Zn-mb), Ni(II) (Ni-mb), Mn(II) (Mn-mb), or Co(II) (Co-mb), collected on a Dianion HP-20 column, washed with 5 column volumes of H₂O, then eluted and freeze dried. Standard variance was equal to or less than 20%.

<table>
<thead>
<tr>
<th>Metal</th>
<th>mb</th>
<th>Cu-mb</th>
<th>Cd-mb</th>
<th>Zn-mb</th>
<th>Ni-mb</th>
<th>Mn-mb</th>
<th>Co-mb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu(II)</td>
<td>0.0376</td>
<td>1.552</td>
<td>bd</td>
<td>0.0014</td>
<td>0.0010</td>
<td>0.0002</td>
<td>bd</td>
</tr>
<tr>
<td>Cd(II)</td>
<td>0.003</td>
<td>0.0006</td>
<td>0.6079</td>
<td>bd</td>
<td>bd</td>
<td>bd</td>
<td>0.0008</td>
</tr>
<tr>
<td>Zn(II)</td>
<td>0.001</td>
<td>bd*</td>
<td>bd</td>
<td>0.6575</td>
<td>0.0017</td>
<td>0.0040</td>
<td>bd</td>
</tr>
<tr>
<td>Ni(II)</td>
<td>0.0001</td>
<td>0.0029</td>
<td>0.0005</td>
<td>0.0002</td>
<td>0.7603</td>
<td>0.0004</td>
<td>0.0107</td>
</tr>
<tr>
<td>Mn(II)</td>
<td>0.0004</td>
<td>bd</td>
<td>bd</td>
<td>0.0028</td>
<td>0.0014</td>
<td>0.6778</td>
<td>0.0003</td>
</tr>
<tr>
<td>Co(II)</td>
<td>0.0003</td>
<td>0.0016</td>
<td>0.0017</td>
<td>0.0004</td>
<td>0.0002</td>
<td>0.0001</td>
<td>0.8068</td>
</tr>
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</table>

*below detection
Table 3: Change in emission intensities from Tyr, following excitation at 280 nm ($\lambda_{ex}$280 nm), THI following excitation at 394 nm ($\lambda_{ex}$394 nm), and HTI following excitation at 340 nm ($\lambda_{ex}$340 nm) following the addition of equimolar concentrations of metals to mb.

<table>
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<tr>
<th>Metal</th>
<th>Change in Emission Intensity</th>
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<tr>
<td></td>
<td>Tyr</td>
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<tr>
<td></td>
<td>310 nm</td>
</tr>
<tr>
<td><strong>Group A</strong></td>
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</tr>
<tr>
<td>Cu(II)$^1$</td>
<td>-4.88$^2$</td>
</tr>
<tr>
<td></td>
<td>-1.00$^3$</td>
</tr>
<tr>
<td>Hg(II)</td>
<td>-4.26</td>
</tr>
<tr>
<td>Au(III)</td>
<td>-6.36</td>
</tr>
<tr>
<td><strong>Group B</strong></td>
<td></td>
</tr>
<tr>
<td>Cd(II)</td>
<td>-0.43</td>
</tr>
<tr>
<td>Co(II)</td>
<td>-0.10</td>
</tr>
<tr>
<td>Fe(III)</td>
<td>-4.84</td>
</tr>
<tr>
<td>Ni(II)</td>
<td>-2.85</td>
</tr>
<tr>
<td>Zn(II)</td>
<td>-0.88</td>
</tr>
<tr>
<td>Mn(II)</td>
<td>+0.17</td>
</tr>
</tbody>
</table>

$^1$from Choi *et al.* [8]

$^2$as isolated by Choi *et al.* [8]

$^3$isolated following Cu(II) saturation and Na$_2$EDTA treatment [8, 10]

$^4$Note: Absence of quenching resulted from the reduction of Cu(II) to Cu(I) before coordination to HTI [8].
Table 4. Thermodynamic parameters as measured by ITC for metal binding to mb.
Thermodynamic parameters for Cu(II) were taken from Choi et al. [8], for comparison purposes
the third binding constant for Cu(II) was not included.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cu(II)*</td>
<td>Ag(I)</td>
</tr>
<tr>
<td>( N_1 ) (metal mb(^{-1}))</td>
<td>0.11 ± 0.003</td>
<td>0.47 ± 0.006</td>
</tr>
<tr>
<td>( K_1 ) (M(^{-1}))</td>
<td>3 x 10(^{14}) ± 3 x 10(^{11})</td>
<td>2.6 ± 0.4 x 10(^7)</td>
</tr>
<tr>
<td>( \Delta H_1 ) (kcal mol(^{-1}))</td>
<td>-146</td>
<td>-2.0 ± 0.2 x 10(^4)</td>
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<tr>
<td>( \Delta S_1 ) (cal mol(^{-1})deg(^{-1}))</td>
<td>-331</td>
<td>-32.9</td>
</tr>
<tr>
<td>( \Delta G_1 ) (kcal mol(^{-1}))</td>
<td>-47.2</td>
<td>-10.1</td>
</tr>
<tr>
<td></td>
<td>Cu(II)*</td>
<td>Cd(II)</td>
</tr>
<tr>
<td>( N_1 ) (metal mb(^{-1}))</td>
<td>0.14 ± 0.001</td>
<td>0.75 ± 0.007</td>
</tr>
<tr>
<td>( K_1 ) (M(^{-1}))</td>
<td>2.6 ± 0.5 x 10(^8)</td>
<td>4.7 ± 0.45 x 10(^4)</td>
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<tr>
<td>( \Delta H_1 ) (kcal mol(^{-1}))</td>
<td>-28.1 ± 0.11</td>
<td>-1.1 ± 0.23 x 10(^4)</td>
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<tr>
<td>( \Delta S_1 ) (cal mol(^{-1})deg(^{-1}))</td>
<td>-55.6</td>
<td>-9.53</td>
</tr>
<tr>
<td>( \Delta G_1 ) (kcal mol(^{-1}))</td>
<td>-11.46</td>
<td>-7.74</td>
</tr>
<tr>
<td>( \chi^2 )</td>
<td>6.47 x 10(^4)</td>
<td>7.14 x 10(^4)</td>
</tr>
</tbody>
</table>
Figure 1. Model for the binding of group A (Ag(I), Au(III), Cu(II), Hg(II), Pb(II) and U(VI)) and group B (Cd(II), Co(II), Fe(III), Ni(II), and Zn(II)) metals by mb. Mb is represented as two bars ending in the N atom of each imidazolate and the S atom from the thiocarbonyl group on 4-thiocarbonyl-5-hydroxy imidazolate (THI; yellow and orange bar) and on 4-hydroxy-5-thiocarbonyl imidazolate (HTI; orange bar). Abbreviations: M, metal in the oxidation state added to mb solutions, and M', metal reduced by mb.
Figure 2. (A) UV-visible absorption spectra of mb following addition of 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 Au(III) per mb. Arrows indicate the direction of spectra changes upon Au(III) additions. (B) Absorption changes at 394 (△), 363 (●), 340 (○), and 302 (■) following 0.1 to 2.0 Au(III) additions. Due to the development of strong absorption/light scattering below 300 nm with 1.1 to 2.0 Au(III) additions, absorption changes in this region could not be monitored (shown in insertion in panel A). (C) CD spectra of mb as isolated (thick line) and following additions of 0.1 to 2.0 molar equivalents of Au(III) (thin lines). (D) The effect of Au(III) addition on the CD spectra at 393 (△), 360 (●), 316 (○), and 202 nm (□). (E) Emission spectra of mb in aqueous solution with different excitation wavelength (nm). $\lambda_{ex} = 280$, 340, and 394 nm at ambient temperature (thick lines). Arrows indicate the direction of spectrum changes upon Au(III) additions and thin lines show the spectra upon completion of changes. (F) Emission intensity changes at 610 ($\lambda_{ex} = 394$nm, △), 461 ($\lambda_{ex} = 340$nm, ●), and 310 nm ($\lambda_{ex} = 280$nm, ■).
Figure 3.  (A) UV-visible absorption spectra of mb following addition of 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.0, 1.25, 1.4, 1.6, 1.8 and 2.0 Ag (I) per mb. Arrows indicate the direction of spectra changes upon Ag(I) additions. (B) Absorption changes at 394 (△), 363 (●), 340 (○), 302 (□), 272 (▲) nm following 0.1 to 2.0 Ag(I) additions. (C) CD spectra of mb as isolated (thick line) and following additions of 0.1 to 2.0 molar equivalents of Au(III) (thin lines). (D) The effect of Au(III) addition on the CD spectra at 404 (◇), 390 (△), 354 (●), 314 (○), and 300 (◆), 283 (■) nm.
Figure 4. (A) UV-visible absorption spectra of mb following addition of 0.1, 0.2, 0.3, 0.4, 0.6, 0.8 and 1.0 Ni(II) per mb. Arrows indicate the direction of spectra changes upon Ni(II) additions. (B) Absorption changes at 394 (△), 340 (●), 302 (○), 264 (■), and 254 nm (□) following Ni(II) additions. (C) CD spectra of mb as isolated (thick line) and following additions of 0.1 to 1.0 molar equivalents of Ni(II) (thin lines). (D) The effect of Ni(II) addition on the CD spectra at 371 (△), 342 (●), 306 (○), 217 (■), and 190 nm (□). (E) Emission spectra of mb in aqueous solution with different excitation wavelength (nm). \( \lambda_{\text{ex}} = 280, 340, \) and 394 nm at ambient temperature (thick lines). Arrows indicate the direction of spectrum changes upon Ni(II) additions and thin lines show the spectra upon completion of changes. (F) Emission intensity changes at 610 (\( \lambda_{\text{ex}} = 394\)nm, △), 461 (\( \lambda_{\text{ex}} = 340\)nm, ●), and 310 nm (\( \lambda_{\text{ex}} = 280\)nm, ■).
Figure 5. A. Difference fluorescence spectra of mb following the addition of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, or 1.8 molar equivalences of Au(III) minus mb. B, Emission intensity changes at 461 nm (λ<sub>ex</sub> = 394 nm, ○), 421 nm (λ<sub>ex</sub> = 394 nm, ●), and at 441 nm (λ<sub>ex</sub> = 394 nm, □).

Figure 6. X-band EPR spectra at 77 K of mb (concentration 4 mM) following the addition of Fe(III) (A), Mn(II) (B) and Co(II) (C) to mb. Experimental conditions: modulation amplitude, 5 G, modulation frequency, 100 KHz, microwave power, 5 mW, temperature 77 K.
Figure 7. (A) Gold X-ray photoelectric spectra of mb at gold to mb molar ratios between 0.05 and 10 Au per mb. (B) Corrected signal from Au(0) (O) and Au(III) (●) at different gold:mb molar ratios.

Figure 8. Transmission electron micrographs of methanobactin solutions following the addition of 1 (A), 1.5 (B), or 2 (C) Au per mb, samples were dried on formvar-coated Ni grids. D, TEM of 2 Au per mb following one freeze-thaw cycle, samples were dried on formvar-coated Ni grids. TEM of 5 (E) and 10 (F) Au per mb samples dried on formvar-coated Cu grids.
Figure 9. (A) Sulfur XPS of mb at gold to mb molar ratios between 0.05 and 0.5 Au per mb. (B) Corrected signal from Cys and Met S (Δ) and thiocarbonyl S (O) at different gold:mb molar ratios. Scale on the right is for signal intensity at 163.3 eV and the scale on the left axis is for signal intensity at 161.4 eV. Arrows indicate the direction of spectrum changes upon Au(III) additions.

Figure 10. Binding isotherm of 3.2 mM HgCl$_2$ (A), HAuCl$_4$ (B), AgNO$_3$ (C), NiCl$_2$ (D), ZnCl$_2$ (E), or FeCl$_3$ (F) into 400 μM mb (cell) aqueous solution at 25°C. Binding isotherm of 1.6 mM HAuCl$_4$ (B). The curve fittings for two-site binding algorithm were used.
CHAPTER 6: OXIDASE, SUPEROXIDE DISMUTASE, AND HYDROGEN PEROXIDE REDUCTASE ACTIVITIES OF METHANOBACTIN FROM TYPE I AND TYPE II METHANOTROPHS

Dong W. Choi, Jeremy D. Semrau, William E. Antholine, Marcus T. McEllistrem, Arlene M. de la Mora, and Alan A. DiSpirito

Abstract

Methanobactin (mb) is a copper binding chromopeptide that appears to be involved in oxidation of methane by the membrane associated methane monooxygenase (pMMO). To examine this potential physiological role the redox and catalytic properties of mb from three different methanotrophs were examined in the absence and presence of O₂. Metal free mb from the type II methanotroph Methylosinus trichosporium OB3b, but not from the type I methanotrophs Methylococcus capsulatus Bath and Methylomicrobium album BG8, was reduced by a variety of reductants, including NADH and duroquinol, and catalyzed the reduction of O₂ to O₂⁻. Copper containing mb (Cu-mb) from all three methanotrophs also showed reductant dependent oxidase activity. Cu-mb also catalyzed the dismutation of O₂⁻ to H₂O₂, and the reductant dependent reduction of H₂O₂ to H₂O. The superoxide dismutase-like and hydrogen peroxide reductase activities of Cu-mb were 4 and 1 order(s) of magnitude higher, respectively, than the oxidase rate. In general, the rates of each reaction were equal to or greater than that observed with free Cu. The results demonstrate that Cu-mb from all three methanotrophs are redox active
molecules and oxygen radical scavengers, with the capacity to detoxify both superoxide and hydrogen peroxide without the formation of the hydroxyl radicals associated with Fenton reactions.

As previously observed with Cu-mb from *Ms. trichosporium* OB3b, Cu-mb from both type I methanotrophs stimulated pMMO activity. However, in contrast to previous studies using mb from *Ms. trichosporium* OB3b, pMMO activity was not inhibited by mb from the two type I methanotrophs at low copper to mb ratios.

**Introduction**

Methanobactin (mb) is a copper binding chromopeptide found in both the extracellular and membrane fractions of many if not all aerobic methane oxidizing bacteria or methanotrophs [1-7]. The crystal structure of copper containing mb (Cu-mb) from *Methylosinus trichosporium* OB3b showed the molecule represented a new class of metal binding compounds with a primary sequence of \( N – 2 - \text{isopropylester} - (4 - \text{thiocarbonyl} – 5 - \text{hydroxy imidazolate}) – \text{Gly} – \text{Ser} – \text{Cys} – \text{Tyr} – \text{pyrrolidine} - (4 – \text{hydroxy} – 5 – \text{thiocarbonyl} - \text{imidazolate}) – \text{Ser} – \text{Cys} - \text{Met} [6]. \) Copper coordination was also unique with a dual S, and N coordination by 4-thiocarbonyl-5-hydroxy imidazolate (THI) and 4-hydroxy-5-thiocarbonyl imidazolate (HTI) [6]. Recent studies have also suggested mb is a dynamic molecule in solution and appears to initially bind Cu(II) as a multimer, probably a tetramer, via THI and Tyr [2,3]. This initial binding is followed by a reduction to Cu(I) then by coordination to HTI. Studies on the metal binding as well as on the solution and thermodynamic properties of mb from *Ms.*
*trichosporium* OB3b suggest the physiological function of mb is that of a copper siderophore or chalkophore [2-9].

In addition to the extracellular fraction, Cu-mb is also observed in the cell membrane fraction [7]. In fact, Cu-mb was initially identified in association with the membrane-associated methane monooxygenase (pMMO) and was originally proposed as a cofactor of the hydroxylase component of the pMMO (pMMO-H) [1,7,10]. In this model, the pMMO was a complex composed of three polypeptides (pmoA, pmoB, and pmoC), i.e. the hydroxyase component (pMMO-H) plus 5 to 8 Cu-mb [1,7,10,11]. Subsequent studies in other laboratories have reported active preparations of pMMO-H with no evidence of Cu-mb [12-19]. The reported activities of pMMO-H in the absence of Cu-mb, however, were low, 2 - 25 % of the reported activities for pMMO-H isolated with Cu-mb. Thus, the role, of Cu-mb in the oxidation of methane to methanol by the pMMO remains a question [1,7,10].

In addition to co-purification, the culture conditions used to stabilize cell free pMMO activity also suggest an association between Cu-mb and pMMO [1,10]. The high copper conditions used to stabilize the pMMO results in increased concentrations of membrane-associated Cu-mb. Cu-mb has also been shown to increase electron flow to the type II Cu(II) centers of pMMO-H and to have superoxide dismutase activity suggesting a secondary roles of Cu-mb in methane catalysis by the pMMO [1,10]. To examine the potential of mb in pMMO-H stabilization or in electron flow to pMMO-H, the oxidase, superoxide dismutase, and hydrogen peroxide reductase activities of mb was examined and compared to the effect of mb on pMMO-H.
Experimental

Organisms, culture conditions and isolation of membrane fraction.

Culture conditions for the isolation of mb from the spent media of *Methylosinus trichosporium* OB3b were described previously [3]. Similar cultivation conditions were used for the isolation of mb from, *Methylococcus capsulatus* Bath, and from *Methylomicrobium album* BG8. Highest yields of mb in the spent media of all three methanotrophs were obtained from 0.2 µM CuSO₄ amended nitrate minimal salts media (NMS), but the yields varied with *Ms. trichosporium* OB3b showing the highest concentrations (35 – 60 mg/l), followed by *Mc. capsulatus* Bath (18 to 24 mg/l), then by *Mm. album* BG8 (1 – 10 mg/l).

For the isolation of the washed membrane fraction of *Mc. capsulatus* Bath, cells were cultured in a batch reactor with a final CuSO₄ concentration of 80 µM as previously described [10].

Isolation of mb.

Methanobactin was prepared from the spent medium of *Ms. trichosporium* OB3b, *Mc. capsulatus* Bath and *Mm. album* BG8 as previously described for *Ms. trichosporium* OB3b [3,10].

Superoxide dismutase (SOD) activity assay

Superoxide anion radical (O₂⁻) was generated using phenazine methosulfate (PMS) and the reduction of nitroblue tetrazolium (NBT) to blue formazan dye was used
as an indicator of $O_2^-$ production as described by Ramadan and El-Naggar [20] with the following modifications. The reactions were initiated by the addition NADH (200 µM final concentration) or duroquinol to an aqueous solution of PMS (6 µM final concentration), NBT (100 µM final concentration), and mb (10 µM final concentration). NADH and duroquinol stock solutions were prepared as previously described [1]. The copper to mb ratio varied between 0.01, 0.1, 0.25, 0.5, 0.75, 1.0, 1.5, or 2.0 copper per mb and the reaction mixture buffered with 10mM MOPS (final volume of 1ml), pH 7.3. Copper controls were measured with equimolar CuSO$_4$ to corresponding copper to mb ratios. Rates of superoxide quenching were monitored by the rate of NBT reduction using the molar extinction coefficient of 15mM$^{-1}$cm$^{-1}$ [21].

**Anaerobic NBT reduction.**

Direct reduction of NBT by mb was monitored under anaerobic conditions using NADH as a reductant. Reagents were prepared in a Coy anaerobic chamber (95% Argon, 5% Hydrogen) and checked for oxygen contamination with anaerobic indicator strips (Oxoid Ltd., Hampshire, UK) prior to use. Duplicate anaerobic reaction mixtures containing 100 µM NBT and 10 µM mb were prepared in anaerobic cuvettes. Reactions were initiated by addition of an anoxic NADH solution with a gas-tight syringe. After a 10 min incubation at room temperature, one of the duplicates was purged with air to introduce $O_2$. 
Oxidase and hydrogen peroxide reductase (HPR) activities.

Reductant dependent oxidase activity was monitored by oxygen consumption rates at room temperature. Reactions were initiated by adding NADH (0.5mM final concentration) or duroquinol (0.5mM final concentration) to reaction mixtures containing either 50 or 100 µM mb. Oxygen concentrations were monitored with either a YSI model 5300 biological oxygen monitor (Yellow Springs Instruments Co., Yellow Springs, OH), a ISO2 oxygen monitor equipped with a 2 mm diameter OXELP electrode (World Precision Instruments Inc., Sarasota, FL), and/or by the fluorescence based oxygen sensing system (Ocean optics Inc., Dunedin, FL). The presence of hydrogen peroxide in the reaction mixture was determined by the addition of catalase (20 – 400 nM final concentration).

HPR activity was also determined by the reduction of catalase activity in reaction mixture containing 0.5mM NADH, 10 nM catalase, and 50 µM mb at room temperature. The reaction mixture in the 1ml oxygen sensing chamber was purged with argon until stable anaerobic base readings were achieved, then H₂O₂ (2 mM final concentration) was injected to initiate the reaction. Oxygen evolution rate from the catalase reaction was measured and the reduction of oxygen evolution rate in the presence of mb was considered as HPR activity. Data were analyzed using linear fitting in Igor Pro 5.03 (Wavemetrics Inc., Lake Oswego, OR).

pH changes associated with HPR activities by Cu²⁺ and Cu-mb were monitored in separate reaction mixtures. In reaction mixtures examining HPR activity by Cu²⁺, duplicate reaction mixtures containing 58 mg/ml bovine serum albumin were used to approximate the buffering capacity of mb.
Methane oxidation activity, protein determination, electron paramagnetic resonance (EPR) spectroscopy, UV-visible absorption spectroscopy, and metal analysis.

Methane oxidation activity, protein, EPR spectroscopy, copper and iron determinations were carried out as described previously [1,3,10,22]. Copper binding capacity of mb from *Ms. trichosporium* OB3b was examined by incubation of mb in the presence of a molar excess of CuCl₂ for 5 min followed by isolation using Sep-Pac cartridges as previously described [1].

Molecular-mass determinations.

Molecular masses of mb samples from *Ms. trichosporium* OB3b were determined on an Agilent Technologies Model 6210 Time-of-Flight Liquid Chromatograph/Mass Spectrometer (TOF LC/MS) using electrospray ionization at the interface between LC and MS. Exact masses (± 5 ppm) were determined for mb as a negative ion using four co-ionized calibration compounds.

X-ray photoelectron spectroscopy (XPS).

XPS was performed on a model Phoibos-150 hemispherical analyzer (SPECS Scientific Instruments, Sarasota, FL) as previously described [23], using an Al Ka X-ray source operated at 150W. To estimate X-ray-induced reduction of Cu(II) in Cu/mb complexes, samples were dried onto graphite substrates, introduced into the instrument and aligned by monitoring the N XPS. Total X-ray exposure time was monitored, and the Cu 2p₃/₂ was measured by scanning from 920 to 950 BE; 20 or 30 scans were signal-
averaged. Spectra were fit to two peaks (using CasaXPS software), and the area of Cu(I) and Cu(II) determined as a function of X-ray exposure time. The Cu(II) areas were normalized against the total XPS area for Cu and scaled to the experimental Cu/mb ratio (based on solution stoichiometry).

Results and discussion

Effect of mb on pMMO-H activity.

The effects of mb from the type I methanotrophs Mc. capsulatus Bath and Mm. album BG8 and from the type II methanotroph Ms. trichosporium OB3b on pMMO activity in the washed membrane fraction of Mc. capsulatus Bath varied with the reductant, with the source of mb, as well as with the Cu to mb molar ratio (Fig. 1). When NADH was used as a reductant, mb from all three methanotrophs stimulated pMMO activity regardless of the Cu to mb ratio (Fig. 1A). Mb is a copper-chelator or chalkophore [3,6] so the final copper to mb following addition to mb to the washed membrane fraction is difficult to determine. However, with the exception of mb from Mc. capsulatus Bath, stimulation was maximal at or near the predicted ratio for the dimer or monomer copper coordination state [3], followed by a decreasing at higher Cu mb ratios.

When duroqunol was used as the reductant, the effect of mb on pMMO activity in the washed membrane fraction from Mc. capsulatus Bath was more complex (Fig.1B). As previously observed [10], mb from Ms trichosporium OB3b stimulated pMMO activity at Cu to mb ratios ≥1 Cu per mb but was inhibitory at lower Cu to mb ratios. In contrast, little to no inhibition at low Cu to mb ratios was observed with the mb from the
two type I methanotrophs with optimal stimulation of pMMO activity between 0.5 and 1 Cu per mb.

**Superoxide dismutase-like activity.**

Cu-mb has been shown to have superoxide dismutase-like (SOD) [10]. To determine if the effects of mb on pMMO activity was related to SOD-like activity, SOD activity by mb was determined at different Cu to mb ratios (Fig. 2). The results showed a relationship between these two activities (Fig. 2, table 1). In fact, negative SOD activities were observed with mb from *Ms. trichosporium* OB3b at the Cu to mb ratios found to be inhibitory to pMMO activity (Fig. 1B and 2). The unexpected negative SOD activities suggested mb either reduced O$_2$ to O$_2^-$ or nitroblue tetrazolium (NBT) to insoluble formazan.

**Oxidase activity by mb**

To determine if mb reduced O$_2$ to O$_2^-$, oxygen uptake rates were monitored at different Cu to mb ratios (Figs. 3A and 4). The mb from all three mb samples showed Cu-dependent oxidase activity with either NADH or duroquinol as a reductant. The mb from *Ms. trichosporium* OB3b also showed Cu-independent oxidase activity. When NADH was used as a reductant with mb from *Ms. trichosporium* OB3b, highest oxidase activities were observed at high and low Cu to mb ratios, and lowest activity at Cu to mb ratio of 0.5 Cu per mb. However, when duroquinol was used as a reductant, and inverse
response to Cu was observed with the highest oxidase activity was observed at 0.5 Cu per mb.

Catalase was added to oxidase reaction mixtures under steady state conditions to determine if hydrogen peroxide was an end product of the oxidase reactions by mb. At low Cu to mb ratios, the addition of catalase to the reaction mixture resulted in the release of 0.5 O$_2$ for every O$_2$ consumed (Fig. 3, 5 & 6). The ratio of O$_2$ consumed verses O$_2$ released following catalase addition under low Cu to mb ratios were similar if not identical to the predicted values for a one electron reduction of O$_2$ (equation 2) followed by the chemical or biological dismutation of superoxide to hydrogen peroxide (equation 3).

1. $XH_2 + mb \rightarrow X^{2+} + mb^-^2$

2. $mb^-^2 + 2O_2 \rightarrow 2O_2^- + mb$

3. $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$

4. Catalase Reaction: $H_2O_2 \rightarrow H_2O + 0.5O_2$

At higher Cu to mb ratios, the O$_2$ released following catalase addition was less than the predicted 0.5 O$_2$ produced per O$_2$ consumed (Fig. 5). As described below, reductant dependent hydrogen peroxide reductase (HPR) activity of mb accounted for the difference between the predicted and observed O$_2$ evolution following catalase addition.

Unexpectedly, the oxidase activities by mb from *Ms. trichosporium* OB3b at different Cu to mb ratios correlated with the UV-visible absorption spectral changes in the 245 to 305 nm range at different copper to mb ratios (Fig 7). As described previously [3], the effect of copper on the UV-visible absorption spectra of mb from *Ms. trichosporium* OB3b in the 240 to 305 nm was complex (Fig. 7A). The UV absorption at
254 nm have been associated the two Cys moieties [3,5] although the possibility of a charge-transfer absorptions following deprotonation of nitrogens [24-26] or with a Cys-S-Cu(I) ligand to metal charge transfer [27-31] could not be ruled out [3]. The UV-visible absorption changes at 282 and 302 nm were originally associated with the phenolic and phenoxy ion forms of Tyr [3]. However, subsequent studies have shown that absorption 302 nm could be altered without an associated change in absorption at 282 nm, suggesting the absorption maxima 282 and 302 nm do not represent the phenolic and phenoxy ion forms of Tyr [2]. The correlations between oxidase activity and absorption changes at 254, 282 and 302 nm suggest the absorption changes may be associated with a Tyr-imidazolate cross link [32-36]. The coupled changes at 254 and 302 nm would be consistent with the absorption changes associated with an extended pi-system between the phenol ring of Tyr and imidazolate ring [34,37]. The crystal structure of Cu-mb isolated following exposure to high, 1000 fold excess, copper concentration [6] shows the Tyr is in the same plane as HTI, but 3.5 Å from the HTI-imidazolate, suggesting if the covalent link did exist it was broken during the isolation and/or crystallization procedure.

**Reduction of nitroblue tetrazolium by mb.**

Although capable of generating superoxide, the rates of oxidase activity were too slow to account for the increased rates of superoxide production observed with mb from *Ms trichosporium* OB3b at low Cu to mb ratios (Figs. 2 and 3) suggesting direct NBT reduction by mb. To exam this possibility, NBT reduction was monitored under anaerobic conditions (Fig. 3B). Consistent with direct dye reduction, reactions initiated
under anaerobic conditions were identical to those monitored under aerobic conditions and the rates comparable to the increased rates of dye reduction observed in the SOD experiments (Fig. 2). Unexpectedly, NBT reduction under anaerobic conditions stopped in anaerobic samples pulsed with dioxygen. Oxygen consumption was observed in pulsed samples suggesting a change of electron acceptor from NBT to dioxygen.

**Hydrogen peroxide reductase activity.**

Hydrogen peroxide reductase activity by mb or Cu-mb was monitored by the quenching of the rate of oxygen evolution by catalase (Fig. 8). As observed with oxidase activity, the mb from both type I methanotrophs followed the general trend of copper dependent HPR activity, while the mb from *Ms. trichosporium* OB3b showed highest HPR activities at copper concentrations less than or greater than 0.5 Cu per mb. To determine if the HPR activities of Cu-mb differed from free copper, the dissolved oxygen concentration and pH changes were monitored in the presence and absence of a reductant (Fig. 9). In the absence of a reduction, the addition of H$_2$O$_2$ to a CuCl$_2$ solution resulted in the production of one proton and 0.5 dioxygen per H$_2$O$_2$ oxidized, which is consistent with equations 5 – 10.

5) $\text{Cu}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Cu}^+ + \text{O}_2\text{H}^+ + \text{H}^+$

6) $\text{Cu}^{2+} + \text{O}_2\text{H}^+ \rightarrow \text{Cu}^+ + \text{O}_2 + \text{H}^+$

7) $\text{Cu}^+ + \text{H}_2\text{O}_2 \rightarrow \text{Cu}^{2+} + \text{OH}^+ + \text{OH}^-$

8) $\text{Cu}^+ + \text{OH}^+ + \text{H}^+ \rightarrow \text{Cu}^{2+} + \text{H}_2\text{O}$

9) $\text{OH}^- + \text{H}^+ \rightarrow \text{H}_2\text{O}$
10) sum: $2\text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}_2\text{O} + 2\text{H}^+$

Oxygen evolution stopped following the addition of a reductant to free copper, with an associated increase in pH. Although the result is not consistent with the equations below (equations 11-15), pH increase can be explained by the presence of highly reactive hydroxyl radical (equation 13) resulting in continuous proton consumption in reaction mixture.

11) $2\text{Cu}^{2+} + \text{NADH} + \text{H}^+ \rightarrow 2\text{Cu}^+ + \text{NAD}^+ + 2\text{H}^+$

12) $\text{Cu}^+ + \text{H}_2\text{O}_2 \rightarrow \text{Cu}^{2+} + \text{OH}^- + \text{OH}^-$

13) $\text{Cu}^+ + \text{OH}^- + \text{H}^+ \rightarrow \text{Cu}^{2+} + \text{H}_2\text{O}$

14) $\text{OH}^- + \text{H}^+ \rightarrow \text{H}_2\text{O}$

15) sum: $\text{H}_2\text{O}_2 + \text{NADH} + \text{H}^+ \rightarrow 2\text{H}_2\text{O} + \text{NAD}^+$

In contrast to free copper, in the absence of a reductant, no oxygen evolution was observed in reaction mixtures containing with Cu-mb and H$_2$O$_2$. In the presence of a reductant, oxygen consumption was observed following the addition of H$_2$O$_2$ to Cu-mb (Fig. 9A). However, no pH changes were observed following the addition of H$_2$O$_2$, which is consistent with the reactions outlined in equations 16 – 17 (Fig. 9B).

16) $2\text{Cu-mb} + \text{NADH} + \text{H}^+ \rightarrow 2\text{Cu-mb}^- + \text{NAD}^+ + 2\text{H}^+$

17) $2\text{Cu-mb}^- + \text{H}_2\text{O}_2 + 2\text{H}^+ \rightarrow 2\text{Cu-mb} + 2\text{H}_2\text{O}$

18) sum: $\text{H}_2\text{O}_2 + \text{NADH} + \text{H}^+ \rightarrow \text{NAD}^+ + 2\text{H}_2\text{O}$

The results suggest that H$_2$O$_2$ reduction by Cu-mb proceeds in the absence of reactive oxygen species associated with free copper. As a control, bovine serum albumin was added to free copper reaction mixtures at equal (wt/vol) concentrations to that of mb
used in the Cu-mb reaction mixture. The bovine serum albumin and copper containing reaction mixtures showed similar if not identical pH changes to the free copper reaction mixtures suggesting the absence of a pH change in Cu-mb resulted from a different reaction mechanism, not due to the buffering capacity of mb.

**Cu²⁺ binding and reduction.**

Previously, we reported XPS results demonstrating that mb will reduce Cu(II) to Cu(I) upon binding the metal ion [3]. Initial efforts to quantitate the number of Cu(II) ions reduced by mb lead to inconsistent results. This was thought to possibly arise from the reduction of Cu(II) to Cu(I) by secondary electrons generated in the sample from the X-ray source as previously reported for transition metal. A cursory examination indicated minimal such reduction, but follow-up studies showed that such reduction does indeed occur. An example of such reduction for Cu/mb of 5.1 is shown in Figure 10, wherein spectra were acquired for the same sample after 7, 27, and 71 m of X-ray exposure (Al Kα, 150W). The results show a loss of intensity in the Cu(II) line with the corresponding increase in Cu(I). Further changes that could be attributed to the reduction of Cu(I) to Cu(0) was never observed regardless of X-ray exposure. This X-ray-induced reduction created a problem in measuring the amount of mb-induced reduction occurs. Experiments were therefore undertaken to factor out the instrument-induced systematic error by measuring copper spectra over the course of one to two hours (described below).

If the number of secondary electrons excited by the X-ray source are assumed to be constant, the decreasing peak area for Cu(II) followed pseudo-first-order kinetics. In
order to measure the loss of Cu(II), the Cu 2p$_{3/2}$ spectra were fit to two peaks (using the program CasaXPS) and the area of Cu(I) and Cu(II) were determined as a function of X-ray exposure time. Normalizing the measured Cu(II) areas to the total area for all Cu oxidation states and scaling areas to the Cu/mb ratio (based on stoichiometry) permitted the Cu(II) signal decay to be measured. A fit of the data to a single exponential using Igor Pro allowed the determination of Cu(II) in the absence of X-ray irradiation at t=0. The loss of Cu(II) as a function of time at Cu/mb of 5.1, is shown in Figure 10 B. Following correction, the results showed that the mb from *Ms. trichosporium* OB3b will reduce approximately 2 Cu$^{2+}$ to 2 Cu$^+$, but only when exposed to Cu$^{2+}$ concentrations > 2 Cu$^{2+}$ per mb (Fig. 11). In general the corrected results are similar to previous studies [3,6], and suggest that either the displacement of Cu$^+$ bound to mb with Cu$^{2+}$ or the presence of multiple Cu$^{2+}$ binding sites.

**EPR spectroscopy.**

In contrast to copper coordination determined in the crystal structure [6], the EPR spectra of mb from *Ms. trichosporium* OB3b suggest EPR-detectable Cu coordination of 2N2O or 3N1O, with no detectable sulfur coordination (Fig. 12). The quality of the signals was quite good as indicated by the resolution of $^{65}$Cu shoulder from the $^{63}$Cu signal. The improved quality of mb samples was the result of improvements in the purification procedure [10]. Combined with the crystal structure of Cu-mb [9], the results suggest a change in copper coordination from 2N2S to 2N2O during oxygen catalysis and may represent the fraction of the population involved in oxygen turnover. The half field signal at g = 1.98 could be either from iron or a copper dimer or both, but is consistent
with a high spin Fe-S signal (Fig.12). Since only 5 to 25% of the copper associated with mb is Cu$^{2+}$, 5 to 10 mM mb were used in EPR studies and at these concentrations trace iron contamination becomes EPR detectable. Iron concentrations in the samples examined were 0.02 Fe per mb and are consistent with the intensity of the iron-EPR signal. However, the EPR spectra of ferric saturated mb samples only showed a narrowing of the g = 4.3 peak suggesting coordination, but not the g = 1.98 signal observed in these samples.

In an attempt to generate a 2N2S signal, the EPR spectra of Cu-mb were examined under anaerobic conditions. At Cu to mb ratios of 0.5 only a small decrease in the intensity of the Cu and Fe signals were observed following incubation under anaerobic conditions for 30 minutes (Fig. 12, traces a). Anaerobicity of the samples during incubation was verified with resazurin indicator strips. The results suggest oxygen remained bound to 0.5Cu-mb and Cu$^{2+}$ remained oxidized. At higher copper to mb ratios, no EPR active copper or iron was observed suggesting the reduction of both metals.

**Summary and concluding remarks**

In addition to SOD-like activity reported in our earlier studies [1, 10], the results presented here show mb also exhibits reductant dependent-oxidase and -hydrogen peroxide reductase activities. Reductant dependent-oxidase activity of mb can reduce molecular oxygen (O$_2$) to superoxide (O$_2^-$) in the presence of atmospheric oxygen and also can directly reduce NBT to insoluble formazan in the absence of oxygen. Numbers
of studies have shown $\text{O}_2^-$ is formed by accident when molecular oxygen adventitiously withdraws an electron from redox components of respiratory chain that are designed to transfer electrons to other substrates [38-43]. In fact, none of the enzymes that have been characterized are thought to generate $\text{O}_2^-$ as a deliberate, stoichiometric product. Studies have also shown these redox active components were able to directly reduce NBT to insoluble formazan in the absence of oxygen [44-47] like mb indicating mb might also be a redox component of the respiratory chain of methanotrophs. This concept is consistent with the results presented here showing the addition of Cu-mb enhanced pMMO activity in washed membrane fractions.

Inhibitory effects of mb from *Ms. trichosporium* OB3b at low Cu to mb ratios can be explained by the generation of $\text{O}_2^-$ by mb. In *Escherichia coli* [48-51], when respiratory vesicles were incubated in vitro with reductant and atmospheric oxygen, normal electron transport was accompanied by the generation of $\text{O}_2^-$ which in turn caused damages on variety of biomolecules. The superoxide generation was approximated that 0.2% of the consumed oxygen was relased as $\text{O}_2^-$ and 0.4% as $\text{H}_2\text{O}_2$. Considering mb does not show any detectable SOD-like or reductant-dependent HPR activites at low Cu to mb ratios, the inhibitory effects of mb on pMMO activity was probably due to the increased reactive oxygen radical generations by added mb.

In contrast to copper coordination determined in the crystal structure [6], the EPR spectra of mb from *Ms. trichosporium* OB3b suggest EPR-detectable Cu coordination of 2N2O or 3N1O, with no detectable sulfur coordination. This result suggests one potentially important feature of mb, that mb may go through different ligation status of Cu depends on the oxidation status of Cu.
The result presented here implies that mb may be the first identified redox active component of respiratory chain with radical scavenging activities such as SOD-like activity, as well as reductant dependent HPR activity. These radical scavenging activities of mb may play an additional important role protecting the biological molecules including pMMO from radical-mediated damage.

**Abbreviations**

Cu-mb, Methanobactin copper complex;  
EPR, Electron paramagnetic resonance;  
HPR, hydrogen peroxide reductase;  
HTI, 4-hydroxy-5-thiobarbonyl imidazolate;  
Mb, methanobactin;  
MMO, methane monooxygenase;  
NBT, nitroblue tetrazolium;  
P, Pearson correlation;  
pMMO, membrane-associated methane monooxygenase;  
pMMO-H, Hydroxylase component of the membrane-associated methane monooxygenase;  
PMS, phenazine methosulfate;  
r, significance (two-tailed);  
sMMO, soluble methane monooxygenase;  
SOD, Superoxide dismutase;
THI, 4-thiocarbonyl-5-hydroxy imidazolate;
XPS, X-ray photoelectric spectroscopy.

References

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    The Netherlands.


Table 1. Base reaction rates of superoxide dismutase, oxidase and H$_2$O$_2$ activities of mb and of pMOM in the washed membrane fraction from *Mc. capsulatus* Bath grown at 80µM CuSO$_4$ amended media.

<table>
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<th>Reductant</th>
<th>SOD (O$_2$· min$^{-1}$·mb$^{-1}$)</th>
<th>Oxidase (O$_2$·min$^{-1}$·mb$^{-1}$)</th>
<th>HPR (H$_2$O$_2$·min$^{-1}$·mb$^{-1}$)</th>
<th>pMOM (nmol PO$_2$·min$^{-1}$·mg protein$^{-1}$)</th>
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<td>NADH</td>
<td>0.1</td>
<td>5.1</td>
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<td>Duroquinol</td>
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Table 2. Bivariant correlations between copper concentrations, absorption at 254, 282, 302 nm and oxidase activity by mb from *Ms. trichosporium* OB3b.

<table>
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<tr>
<th>Cu/mb</th>
<th>254 nm</th>
<th>282 nm</th>
<th>302 nm</th>
<th>Oxidase</th>
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<tr>
<td>Oxidase</td>
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*Correlation is significant at the 0.05 level (2-tailed).

**Correlation is significant at the 0.01 level (2-tailed).
Table 3. Pearson correlations of pMMO activity in the washed membrane fraction from *Mc. capsulatus* Bath grown at 80 μM CuSO₄ amended media to the oxidase, H₂O₂, reductase and superoxide dismutase activities of mb *Mc capsulatus* Bath (Mc), *Ms trichosporium* OB3b (Mt), and *Mn. album* BG8 (Ma) at different Cu to mb ratios.

<table>
<thead>
<tr>
<th>pMMO</th>
<th>NADH Oxidase</th>
<th>H₂O₂ Reductase</th>
<th>Duroquinol Oxidase</th>
<th>H₂O₂ Reductase</th>
<th>Superoxide Dismutase</th>
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<tbody>
<tr>
<td></td>
<td>Mc Mt Ma</td>
<td>Mc Mt Ma</td>
<td>Mc Mt Ma</td>
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<tr>
<td>Mc</td>
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<td>-0.81</td>
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<tr>
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<td>0.73</td>
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</tr>
<tr>
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<td>0.92</td>
<td>0.99</td>
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<tr>
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<tr>
<td>Mc</td>
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<td>0.45</td>
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<tr>
<td>Ma</td>
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<td>0.84</td>
<td>0.85</td>
<td>0.93</td>
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Numbers in bold: correlations are significant at the 0.05 level (2-tailed).
Numbers in shaded box: correlations are significant at the 0.01 level (2-tailed).
Figure 1. Effect of mb from *Mc. capsulatus* Bath (○), *Ms. trichosporium* OB3b (△) and *Mm. album* BG8 (□) at different Cu to mb ratios on propylene oxidation by the washed membrane fraction of *Mc. capsulatus* Bath using (A) NADH or (B) duroquinol as a reductant. Dotted line marks the pMMO activity of in the washed membrane fraction before the addition of mb.

Figure 2. Superoxide dismutase (SOD) activity of Cu (◇) and of mb from *Mc. capsulatus* Bath (○), *Ms. trichosporium* OB3b (△) and *Mm. album* BG8(□) at different Cu to mb ratios.
Figure 3. A. Oxygen trace as measured with a 2mm diameter OXELP oxygen electron following the addition of NADH and either of mb (○) or Cu-mb (●) from Ms. trichosporium OB3b, followed by the addition of 20, 100, 200 or 400 nM catalase. B. Reduction of nitroblue tetrazolium by Cu-mb from Ms. trichosporium OB3b using NADH as a reductant under aerobic (▲) and anaerobic conditions (▲). Arrow indicates the addition of air to anaerobic reaction mixture.

Figure 4. Oxidase activity of Cu (◇) and of mb from Mc. capsulatus Bath (○), Ms. trichosporium OB3b (△) and Mm. album BG8(□) at different Cu to mb ratios using (A) NADH or (B) duroquinol as a reductant.
Figure 5. Oxygen evolution following the addition of catalase to reaction mixtures containing mb from *Mc. capsulatus* Bath (∗), *Ms. trichosporium* OB3b (∆) and *Mm. album* BG8(□) at different Cu to mb ratios using (A) NADH or (B) duroquinol as a reductant.

Figure 6. Proposed reaction mechanism for oxidase and superoxide dismutase (SOD) activities of mb, Cu-mb, and Cu in the presence of NADH and for the competition between catalase and hydrogen peroxide reductase (HPR) for H₂O₂.
Figure 7. A. UV-visible absorption spectra in the Tyr region of mb from *Ms. trichosporium* OB3b at different Cu to mb ratios. B. Oxidase activity (▲) and UV-visible absorption changes at 254 nm (○), 282 nm (□), and 301 nm (◇).

Figure 8. Hydrogen peroxide reductase activity of Cu (◇) and of mb from *Mc. capsulatus* Bath (○), *Ms. trichosporium* OB3b (△) or *Mm. album* BG8 (□) at different Cu to mb ratios using NADH as a reductant.
Figure 9. A. Oxygen trace of anaerobic solutions of Cu(II) or Cu-mb following the addition of \( \text{H}_2\text{O}_2 \) (1 min), followed by the addition of 400 nM catalase. B. Change in proton concentration in anaerobic solutions of Cu(II) (◆ and ◊), or Cu-mb from either *Mc. capsulatus* Bath (○), *Ms. trichosporium* OB3b (▵) or *Mm. album* BG8(□) following the addition of \( \text{H}_2\text{O}_2 \), with (open circles) or without the addition of NADH.

Figure 10. A. X-ray beam inducted reduction of Cu(II) to Cu(I). B. Decay of Cu(II) as a function of X-ray exposure. Fit of data to exponential decay at \( 3.32^{-0.0148t} \).
Figure 11. Oxidation states of copper at high copper to mb ratios as determined by X-ray photoelectric spectroscopy.

Figure 12. X-band EPR spectra of 5 mM mb containing 0.5 Cu per mb (a) and 0.8 Cu per mb (b) under aerobic and anaerobic conditions.
CHAPTER 7: MÖSSBAUER STUDIES OF THE MEMBRANE-ASSOCIATED METHANE MONOOXYGENASE FROM Methylococcus capsulatus Bath: EVIDENCE FOR A DIIRON CENTER

A paper submitted to Journal of the American Chemical Society

Marlène Martinho, Dong W. Choi, Alan A. DiSpirito, William E. Antholine, Jeremy D. Semrau, and Eckard Münck

Abstract

Two methane monooxygenase (MMO) systems have been identified in methanotrophic bacteria, namely a soluble or cytoplasmic MMO and a membrane associated or particulate MMO. The active site of the well characterized soluble MMO contains a bis-µ-hydroxo bridged diiron cluster. X-ray crystallographic studies of the particulate enzyme, pMMO, have identified two copper centers on the α subunit (pmoB) of the αβγ trimer and a site at the interface of the βγ subunits filled by a Zn, apparently from the crystallization buffer. In our hands, pMMO preparations containing 1-2 iron atoms per αβγ show the highest catalytic activity. We have employed Mössbauer spectroscopy to characterize the iron in our preparations. Interestingly, we find in pMMO a component with the same spectral properties as the antiferromagnetically coupled diiron(III) cluster in the soluble enzyme. In whole cells we find nearly 1 diiron center per αβγ of pMMO; in purified enzyme preparations, only 10% of the sites appear to be...
occupied. These occupancies correlate well with the measured specific activities of purified pMMO and pMMO in whole cells. We suggest that it is the “Zn-site” that accommodates the diiron center in active pMMO.

Introduction

Methane monooxygenase (MMO) catalyzes the energy dependent oxidation of methane to methanol in methanotrophic bacteria.\textsuperscript{1, 2} In these organisms two different methane monooxygenases have been identified, namely a membrane-associated or particulate MMO (pMMO) and a cytoplasmic or soluble MMO (sMMO). In methanotrophs that express both forms of the enzyme, the copper concentration during growth dictates which MMO is expressed.\textsuperscript{2-5} In cells cultured under a low copper/biomass ratio, the sMMO is predominately expressed, whereas cells cultured at higher copper/biomass ratios exclusively express the pMMO (sMMO is not transcribed).\textsuperscript{6-8} The sMMO is a well characterized three-component enzyme consisting of a hydroxylase, a reductase and a regulatory protein.\textsuperscript{9-12} Spectroscopic and X-ray crystallographic studies have established that the hydroxylase contains an oxygen bridged diiron cluster.\textsuperscript{13-16} Here we provide evidence that pMMO contains a diiron cluster as well.

Owing to the low specific activity and instability of most pMMO preparations,\textsuperscript{6, 17-20} comparatively little is known about the molecular properties of this enzyme. As isolated, pMMO is composed of three polypeptides with molecular masses of 45,000, 26,000, and 23,000 Da with a subunit structure of (αβγ)\textsubscript{3}.\textsuperscript{6, 17, 18, 20-22} Most researchers agree each αβγ contains 2 – 3 Cu atoms\textsuperscript{2, 6, 17-20, 23} although one group has suggested that
15 Cu atoms are arranged into catalytic and electron transfer trinuclear copper clusters.\textsuperscript{22, 24, 25} The 2.8 Å resolution crystal structure of pMMO revealed that each αβγ contained a dicopper site, a mononuclear copper site, and a third site occupied by zinc.\textsuperscript{21, 23} However, the preparation used for growing the crystal was inactive and did not contain zinc (which was added to the crystallization buffer).\textsuperscript{21, 23}

**Results and discussion**

The involvement of non-heme iron in methane oxidation by the pMMO has been proposed by some laboratories\textsuperscript{6, 17, 26-29} and disputed by others.\textsuperscript{22, 24, 30} In our laboratory at Iowa State University we have observed that preparations of pMMO showing highest specific activity contain 1–2 iron atoms.\textsuperscript{6} We therefore decided to employ Mössbauer spectroscopy to characterize the iron components. This technique is particularly well suited to explore iron environments that are EPR-silent and optically uninformative in the visible region, as is the spin-coupled diiron(III) center of the hydroxylation component of sMMO. It seemed reasonable to us to search for a similar diiron cluster because this is the only type of center known to oxidize methane to methanol at room temperature.

The 4.2 K Mössbauer spectrum of the antiferromagnetically coupled diiron(III) centers of sMMO consists of a doublet with quadrupole splitting $\Delta E_Q = 1.12$ mm/s for the *M. capsulatus* Bath enzyme\textsuperscript{15, 31} and $\Delta E_Q = 1.02$ mm/s for that of *M. trichosporium* OB3b\textsuperscript{13, 16}; both enzymes have an isomer shift $\delta = 0.50$ mm/s at 4.2 K (The clusters of the two enzymes yield broad absorption lines and equivalent fits have been obtained by assuming different $\Delta E_Q$ values for the two iron sites; see ref 13, 15, 16, 31).
Table 1 lists analytical and activity data of our purified pMMO sample and of whole cells grown at high copper (80 µM) and iron (40 µM); the entries are discussed in the Supporting Information. Figure 1 shows 4.2 K Mössbauer spectra of purified pMMO. The central portion of the 45 mT spectrum (Figure 1A) exhibits two overlapping doublets with $\Delta E_Q(1) = 1.05$ mm/s, $\delta(1) = 0.50$ mm/s ($\approx 20\%$ of total Fe) and $\Delta E_Q(2) = 2.65$ mm/s, $\delta(1) = 1.25$ mm/s ($\approx 18\%$ of total Fe); the $\delta$ value of doublet 2 is characteristic of a high-spin Fe$^{2+}$ with octahedral N/O coordination. The majority of the iron in the spectrum of Figure 1A, perhaps up to 60% of total Fe, belongs to a heterogeneous population of Fe$^{3+}$ species exhibiting magnetic hyperfine structure with splittings up to 17 mm/s Doppler velocity. This Fe$^{3+}$ fraction is EPR silent at X-band (Figure S3), and its $\Delta E_Q$ and $\delta$ values (0.63 mm/s and 0.51 mm/s at 120 K) are the same as those reported for mineralized nanoparticles (attributed to ferric phosphate) in mitochondria of yeast frataxin homolog (Δyfh1) mutants. The $\Delta E_Q$ and $\delta$ values of doublet 1 match those reported for the diiron(III) centers of sMMO. The solid line in Figure 1A is a spectral simulation representing two doublets 1 and 2, drawn such that their sum represents 38% of the total absorption; the features of doublet 1 are indicated by the offset dashed line.

The spectrum of Figure 1B was recorded in an applied magnetic field of 8.0 T. Most interestingly, the 8.0 T spectrum shows that the iron of doublet 1 belongs to a diamagnetic (S=0) center, as demonstrated by the spectral simulation outlined by the vertically displaced solid line. The values of $\Delta E_Q(1)$ and $\delta(1)$, together with the observed diamagnetism of this spectral component, strongly suggest that doublet 1 represents an antiferromagnetically coupled diiron(III) center similar to that found in sMMO.
Mössbauer spectroscopy is a very useful technique for the study of iron-containing proteins in whole cells, provided the concentration of the targeted proteins can be increased by overexpression or by employing special growth conditions. Since *M. capsulatus* Bath produces large amounts of pMMO (≈20% of whole cell protein) when grown at high copper and iron concentrations, we were curious whether doublet 1 would be observed in whole cells. We have found that maximal pMMO activity in cell free fractions requires the addition of approximately 40 µM iron in conjunction with 80 µM copper in the culture media. We have recorded Mössbauer spectra of whole *M. capsulatus* Bath cells grown on media high in copper and iron between 1.5 K and 120 K in applied fields up to 8.0 T. Figures 2B and 2D show two representative 4.2 K spectra. The signal strength of the Mössbauer spectrum of Figure 2B indicates that the cells contain roughly 5 mM $^{57}$Fe, in good agreement with the chemical analysis (5.2 mM). Ca. 40% of the iron belongs to a magnetic component of high-spin Fe$^{3+}$ outlined by the solid line in (B); it yields a doublet with $\Delta E_Q \approx 0.6$ mm/s and $\delta = 0.45$ mm/s at 120 K (not shown). This component exhibits spectra typical of superparamagnetic nanoparticles, probably mineralized excess iron accumulated during aerobic growth at high Fe concentrations. Simulation of the 45 mT spectrum of the superparamagnetic component of Figure 2B is quite straightforward (but not unique); however, fitting the outer four lines fixes position and intensities of the two innermost lines of the six-line pattern. Subtraction of the simulated superparamagnetic component from the raw data yields a spectrum (Figure 2C) which exhibits doublet 1 (≈20%, solid line) with exactly the same parameters as observed in the purified enzyme. Also observed is a high-spin Fe$^{2+}$ species with $\Delta E_Q \approx 3.00$ mm/s and $\delta \approx 1.25$ mm/s. The solid line in the 8.0 T spectrum of Figure
2D is a spectral simulation showing that the iron of doublet 1, as in the purified protein, belongs to a diamagnetic center. The broad features, stretching from ca. –9 mm/s to +9 mm/s Doppler velocity, belong to the superparamagnetic components and the Fe$^{2+}$ species. Finally, Figure 2A shows a 4.2 K Mössbauer spectrum of cells grown at low Cu and $^{57}$Fe concentrations (each 5 μM; pMMO < 5% of total cell protein) where pMMO expression is reduced four fold. Compared to the sample of Figure 2B the signal amplitudes have declined by at least a factor 15, showing that the iron observed in the spectra of Figure 2B and D accumulates when the cells are growing at high Cu and Fe concentrations.

The purified pMMO sample (1.06 mM $\alpha\beta\gamma$) had an iron concentration of 1.2 mM. If we associate the iron of doublet 1 with a diiron(III) cluster, we obtain a site occupancy of ca. 11%, assuming that pMMO can accommodate one diiron center/$\alpha\beta\gamma$. The cells of Figure 2B had 5.2 mM iron, yielding $0.2 \times 5.2 / 2 = 0.52$ mM diiron centers. In previous experiments we estimated that ca. 20% of the protein in cells cultured in media containing on 80 μM Cu and 40 μM Fe belongs to pMMO. Using this estimate, the sample of Figure 2B has 0.56 mM pMMO, suggesting that pMMO in thus cultured cells has $\approx 0.93$ diiron(III) center/$\alpha\beta\gamma$. The above estimates agree quite well with the observation that we recover ca. 10% of activity after purification of the protein.

We have assigned doublet 1 to a diiron(III) center. The Mössbauer properties of doublet 1 are also compatible with those observed for some low-spin ferrous hemes, such as cytochromes c and b, and [4Fe-4S]$^{2+}$ clusters. However, the UV/visible spectrum of the Mössbauer sample (Figure S2) indicates less than 0.005 hemes/$\alpha\beta\gamma$, and the cellular
concentration of heme in cells cultured in 5 µM Cu and 5 µM Fe is essentially identical
to cells cultured in 80 µM Cu and 40 µM Fe.\textsuperscript{20} [4Fe-4S]\textsuperscript{2+} clusters can be excluded by
observing that pMMO has only one cysteine residue, Cys 92 on the α subunit,\textsuperscript{21, 36} and
that the presence of sulfide has not been reported by any laboratory.

The Fe\textsuperscript{2+} component(s) observed in whole cells have a larger $\Delta E_Q$ than the Fe\textsuperscript{2+}
species observed in Figure 1A, and thus represent a different type of Fe\textsuperscript{2+}, plausibly iron
bound to transporters and storage components. Perhaps as much as 60% of the iron
observed in the purified pMMO sample of Figure 1 belongs to high-spin Fe\textsuperscript{3+}. The EPR
spectra of Figure S3 are almost devoid of iron associated signals; the resonances at $g \approx 6$
and 4.3 account each for at most 10 µM Fe\textsuperscript{3+}, i. e. only 1% of the iron. We suspect that
the EPR-silent Fe\textsuperscript{3+} represents remnants of the mineralized fraction observed in whole
cells that copurify with the enzyme. We have observed similar EPR-silent Fe\textsuperscript{3+} fractions
in other proteins studied in our laboratory; invariably, these fractions disappeared as
purification procedures improved. The reader may wonder whether doublet 1 could
represent a dimer fraction of a mineralized Fe\textsuperscript{3+}. We have recently studied Mössbauer
spectra of \textit{yah1}-depleted mitochondria from aerobically grown \textit{S. cerevisiae}. For this
mutant (which lacks iron-sulfur proteins) all detectable iron (2-3 mM) could be assigned
to Fe\textsuperscript{3+} nanoparticles, with no evidence for a diamagnetic dimer fraction.\textsuperscript{37}

Given the similarities of the Mössbauer parameters of doublet 1 with those
observed in sMMO, it is reasonable to propose that pMMO has an active site consisting
of a diiron cluster, and that this cluster is bound in the site occupied by Zn from the
crystallization buffer, a possibility indicated by Lieberman and Rosenzweig.\textsuperscript{21}
Interestingly, this site has two conserved His and two conserved carboxylates (His 160
and 173, Asp 156 and Glu 195). Moreover, the site has four additional nearby residues with carboxylates functions, Glu 69, Glu 176, Asp 166 and Asp 168, which are conserved in all known pMMO sequences. Thus, as shown in Figure 3A, the “Zn site” of pMMO has the requisite ligands to accommodate a sMMO type diiron center, and has suggestive similarities with the diiron site of sMMO, shown in Figure 3B. Placing the active site of pMMO into the “Zn site” is supported by the observation that exposure of the cells or purified pMMO to $^{14}$C-acetylene, a suicide substrate for pMMO, yields labeled β subunits (the copper sites of pMMO are located on α).20, 38-40 With a target spectral signature, the loss of iron during purification of pMMO can now be studied by recording Mössbauer spectra through the various steps of the purification procedure.

Acknowledgement

This work was supported by the NIH grant EB-001475 and Department of Energy grant 96ER20237 and by the Office of Vice President for Research at The University of Michigan.

References


35. The magnetic spectra of superparamagnetic particles are exceedingly complex as they depend on the size, shape and shape distribution of these particles, see ref. 34.


Table 1. Properties of whole cells and purified pMMO Mössbauer samples.

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<th>Whole Cells</th>
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<td>Total Fe (mM)</td>
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<td>Diiron(III)/$\alpha \beta \gamma$</td>
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<td>Activity (nmol·min$^{-1}$·mg pMMO protein$^{-1}$)</td>
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Figure 1. 4.2 K Mössbauer spectra of purified pMMO recorded in parallel applied fields of 45 mT (A) and 8.0 T (B). The solid line in (A) is a spectral simulation for doublet 1, assigned to a diamagnetic diiron(III) center (20% of Fe), and a high-spin Fe\(^{2+}\) (18%) component. The remainder of the absorption (magnetic components) belongs to various high-spin Fe\(^{3+}\) species. The dashed line outlines doublet 1. The solid line in (B) is a spectral simulation for the putative diiron(III) center, assuming S=0 and equivalent sites with $\Delta E_Q = +1.05$ mm/s, $\eta = 0.8$ and $\delta = 0.50$ mm/s; $\eta$ is the asymmetry parameter of the electric field gradient tensor. The arrow points at the high energy feature of the high-spin ferrous component.
Figure 2. 4.2 K Mössbauer spectra of *M. capsulatus* Bath cells grown at 5 µM Cu and 5 µM $^{57}$Fe (A) and at 80 µM Cu and 40 µM $^{57}$Fe (B-D). The spectra were recorded in parallel applied fields as indicated. The sample of (B) and (D) consisted of cells grown at 80 µM Cu and 40 µM $^{57}$Fe, then harvested, washed, and resuspended anaerobically. The solid line in (B), representing 40% of the $^{57}$Fe, is a simulation of the superparamagnetic component, obtained by superimposing four high-spin ferric spectra; the simulation was solely aimed at representing the spectral area of this species at one particular applied field. (C) Difference spectrum obtained by subtracting the simulated spectrum of (B) from the raw data of (B). The solid line represents doublet 1 (20% of $^{57}$Fe). (D) 8.0 T spectrum. The solid line is a simulation assuming that the iron of doublet 1 is diamagnetic.
Figure 3. (A) Replacement of the mononuclear Zn by a diiron center in the X-ray structure of pMMO.\textsuperscript{21} (B) Environment of the diiron center in the sMMO from \textit{M. trichosporium} OB3b.\textsuperscript{14}
Supporting Information

Materials and Methods

Organism and cultivation. *M. capsulatus* Bath was cultured on nitrate mineral salts media (NMS) in a 10L fermentor with increasing copper and iron concentration as previously described.\(^1\) During cultivation the cells were sparged at flows rate of 70 ml•min\(^{-1}\) methane and between 400 and 800 ml•min\(^{-1}\) of air. The chemostat was maintained at pH 7.0 \(\pm 0.2\) and oxygen concentrations of 0 – 10% saturation by controlling stirring rate and air flow rates. For cell samples at lower copper concentrations, cells were harvested following one fermentor turnover at 5 µM CuSO\(_4\).

Isolation of cells, membranes and pMMO. Cell, membrane, and pMMO isolations were performed at 0 - 4ºC under anaerobic conditions (95% argon and 5% hydrogen, v/v) as previously described\(^2\) with the omission of the second DEAE-Sepharose column. For the solublization of pMMO, a 10% (wt/vol) solution of dodecyl \(\beta\)-D-maltoside was added with stirring to the washed membrane fraction to final concentrations of 1.2 g of dodecyl \(\beta\)-D-maltoside per g membrane protein.

Preparation of Mössbauer and EPR samples. Anaerobic pMMO samples were prepared in a Coy chamber (atmosphere 95% Ar and 5% H\(_2\)). EPR tubes and Mössbauer cups were filled in the anaerobic chamber. Samples with gas addition were first placed in 6 ml serum vial closed with Teflon lined silicon septa and 2 ml of each gas added via a gas tight syringe. EPR tubes were capped, removed from the chamber, and immediately frozen in liquid nitrogen. Mössbauer cups were filled, placed in Reacti-vials (Pierce,
Rockford, IL), capped with silicon lined Teflon septa, removed from the chamber, frozen in liquid nitrogen, and stored in Reacti-vials.

Washed cell suspension were make anaerobic by four vacuum-purge cycles with Ar then incubated in the anaerobic chamber until the solution tested negative on resazurin anaerobic indicator strips (Oxoid, Basingstoke, Haunts, UK). Cell suspensions were used as isolated, or incubated in closed serum vials with stirring following the addition of, (1) air and CH₄, (2) air, CH₄ or 10 mM formate, or (3) formate (The three samples gave identical Mössbauer spectra). Cell samples were then used to fill EPR tubes, capped and frozen as described above. The remaining cell suspensions were then placed in gas-tight centrifuge tubes and centrifuged at 10,000 X g for 20 m. The cells were then moved to an anaerobic chamber tested and tested for oxygen contamination using anaerobic indicator strips. The cell pellet was scooped into 10 ml syringe and the cell paste layered into Mössbauer cups to a pre-marked 600 µl indicator depth, placed in Reacti-vials, sealed and frozen as described above.

**Enzyme Activity.** MMO activity was determined by the epoxidation of propylene as previously described.² The reductants used were duroquinol (approximately 30 mM) for cell free extracts and formate (2.5 mM) for whole-cell samples. The reductants were prepared and checked as previously described.¹ Reactions were initiated by the addition of 2 ml propylene and 2 ml of air. Reaction mixtures were incubated at 42 °C on a rotary shaker at 250 rpm.

In addition to propylene oxidation activity, the presence of sMMO was monitored by the formation of napthol from naphthalene as described by Brusseau et al.³
**Electrophoresis, protein, and metal determinations.** SDS-denaturing gels, protein and metal determination were determined as previously described.\(^1\) For whole cell Mössbauer samples, metal and protein concentrations were determined from samples taken from Mössbauer cups.

**Spectroscopy.** UV-visible absorption spectra were obtained as previously described.\(^1, 4-7\) Mössbauer spectra were recorded with two spectrometers, using Janis Research Super-Varitemp dewars that allowed studies in applied magnetic fields up to 8.0 T in the temperature range from 1.5 to 200 K. Mössbauer spectral simulations were performed using the WMOSS software package (WEB Research, Edina, MN). Isomer shifts are quoted relative to Fe metal at 298 K. EPR spectra were recorded on a Bruker EPP 300 spectrometer equipped with an Oxford ESR 910 liquid helium cryostat and an Oxford temperature controller. The EPR software package SpinCount was provided by Dr. M. P. Hendrich of Carnegie Mellon University.

**Results**

**Isolation of pMMO.** Purified pMMO preparations with activities above 150 nmol min\(^{-1}\) mg\(^{-1}\) protein can be obtained on a consistent basis following the culture conditions and isolation procedures outlined by Choi et al.\(^1\) One of the key factors in the isolation of higher activity pMMO preparations was to increase the iron concentration in the culture media while increasing the copper concentration. The purified samples showed the \(\alpha\beta\gamma\) subunits of the hydroxylase component of the H-pMMO (Figure S1). The purified enzyme generally contains a number of minor band between the \(\alpha\) and \(\beta\) subunits which
have been shown to be breakdown polypeptides of the α subunit.\(^1, 8, 9\) All purified enzyme preparations also contained 5 to 8 molecules (per \(αβγ\)) of copper containing methanobactin\(^7, 10-12\) which is not apparent on SDS-denaturing gels because of its low molecular mass (1,218 Da) and poor staining properties. The absence of sMMO in pMMO samples was ensured by culturing cells in media containing copper concentrations \(≥\) 5 \(µ\)M as previously described.\(^1\) The absence of sMMO was also verified by testing cell samples for expression of sMMO transcripts,\(^1\) absence of naphthylene oxidation,\(^3\) and examination on SDS-denaturing gels.

**Metal composition and activity of pMMO samples.** The metal composition of purified H-pMMO purified by the procedure of Choi et al.\(^1\) consistently is ca. 2 Cu and 1 - 2 Fe per \(αβγ\). An additional 5 to 8 Cu atoms per \(αβγ\) are found associated with methanobactin. The Dalton and Rosenzweig laboratories have reported similar metal compositions for H-pMMO from *M. capsulatus* Bath.\(^8, 13\) Other groups have reported that Fe is either not present\(^14-16\) or is adventitiously bound to the enzyme.\(^13, 17, 18\) However, the specific activity of the purified pMMO samples containing low iron concentrations are less than 10% of the pMMO activities reported here. Different purification strategies may explain the different iron concentrations between pMMO samples. Research groups that do not observe stochiometric concentrations of iron in pMMO samples purify the pMMO aerobically and from cells that have been freeze-thawed. In our laboratory 80 to 90% of pMMO activity is lost following one freeze-thaw cycle. In cell-free samples, oxygen has been shown to inactivate the pMMO, probably due to the formation of reactive oxygen species. Formation of reactive oxygen species in cell free fractions containing respiratory
components, especially following a sonication step used by some groups reporting the absence of iron, is well documented.\textsuperscript{19-22}

Preparations purified by the procedure described here generally results in trace heme contamination (Figure S2). This heme can be removed by a second DEAE-Sepharose column step.\textsuperscript{1} However, the addition of a second DEAE-Sepharose column to the purification procedure generally lowers the specific activity of the enzyme by 20%. In general, the heme contamination in pMMO samples by the procedure described here accounts for 0.5 – 2% of the iron observed in the final sample. Our pMMO Mössbauer sample might contain a minor ferric cytochrome $c_{555}$ contaminant.\textsuperscript{23} Using the molar extinction coefficient of $\varepsilon_{411} = 164 \text{ mM}^{-1}\text{cm}^{-1}$, the iron associated with cytochrome $c_{555}$ accounts for 0.7% of the total iron in the sample.

\textit{EPR spectra.} The EPR spectra of the pMMO preparation used in this study showed the presence of the type II Cu(II) center characteristic of pMMO preparations.\textsuperscript{1, 4, 6, 8, 10, 13-15, 23-26}

Figure S3 (A) shows an EPR spectrum of an aliquot of the purified Mössbauer sample (1.06 mM $\alpha\beta\gamma$) recorded at 25 K at a microwave power of 20 $\mu$W. Using the software package SpinCount, we have quantified the Cu$^{2+}$ content of the sample against a Cu-EDTA standard by double integration, obtaining a Cu$^{2+}$ spin concentration of 0.82 mM. The spectrum shown in Figure S3 (B) was recorded at 2 mW at 11 K; under these experimental conditions the Cu$^{2+}$ is partially saturated. We have estimated the spin concentrations of the $g = 6$ species (there are two species with $E/D \approx 0$ and $E/D = 0.01$)
and the $g = 4.3$ species by spectral simulations, finding that each group contributes slightly less than 10 $\mu$M spin, i.e. each represents less than 1% of the iron in the sample.

Comments on Mössbauer spectra. Approximately half of the iron, perhaps as much as 60%, observed in whole cells belongs to the component designated as Fe$^{3+}$ nanoparticles. We have not studied this component in great detail, but its blocking temperature is between 10 and 15 K ($T_B$ is the temperature at which 50% of the nanoparticles produce a doublet and 50% exhibit magnetic splittings). The quadrupole splitting associated with this component is $\Delta E_Q \approx 0.6$ mm/s. The broad magnetic component observed in the purified enzyme, ca. 40-60% of the iron, is EPR silent at X-band. We suspect that it represents remnants of nanoparticles that copurify with the enzyme. We have occasionally observed similar components in other projects, and these components invariably vanished as purification became more refined. The observation that the spectra of the Fe$^{3+}$ contaminants in whole cells and purified pMMO differ at low temperature is not incompatible with the notion that they both represent nanoparticles, as the magnetic spectra are size dependent. The Fe$^{3+}$ contaminants have the same $\Delta E_Q$ and $\delta$ at temperatures above 100 K; these values also agree with those attributed to ferric phosphate nanoparticles.

We mention in the text our studies of mutant mitochondria that lack the ability to synthesize iron-sulfur clusters; for these mutants we did not observe any doublet 1 iron. In other projects, we have observed also mineralized iron components in whole cells; in these studies the synthesis of iron-sulfur clusters was not suppressed and diamagnetic doublets attributable to [4Fe-4S]$^{2+}$ clusters were observed.

The high-spin ferrous components in whole cells and purified pMMO have different $\Delta E_Q$ and thus are not good candidates for an iron-based active site. This leaves us with the iron of doublet 1 as a candidate for an iron-based active site. Since $\Delta E_Q$ and $\delta$
of doublet 1 agree with the reported values for sMMO, and since the component is diamagnetic it is reasonable to postulate that it represents a diiron(III) center. The lack of absorption in the visible region of the optical spectra is compatible with this conclusion. Because some low-spin hemes, such as cytochrome c or b, have similar Mössbauer signatures as doublet 1, it is important to exclude the presence of a significant cytochrome contamination. Given the formidable extinction coefficients in the α and β bands of the cytochromes, the optical spectrum of Figure 2 shows that our sample has less than 1% of the iron, if any, associated with low-spin ferrous hemes. Of course, some other low-spin ferrous environment might yield spectra similar to those of doublet 1, but most low spin ferrous environments yield smaller isomer shifts, and exhibit a wide range of quadrupole splittings. Finally, the specific activity, observed in whole cells and purified pMMO correlates with the amount of doublet 1 iron.

Upon completing the Mössbauer studies of Figure 1, we have attempted to reduce the iron of doublet 1 into the ferrous state by incubating the sample for 20 min with six reducing equivalents dithionite and three equivalents of duroquinol under anaerobic conditions. No reduction was observed. It should be noted that pMMO preparations typically loose 90% of their activity in a freeze/thaw cycle, so the material exposed to the reductant would have been essentially inactive. Whatever happens to pMMO in a freeze/thaw cycle, it is not the loss of doublet 1 iron that causes loss of activity. Possibly, a conformational change prevents access of the reductant to the diiron site.

We have to do more systematic cell growth studies, but the following comment may be useful. The pMMO of cells grown on 5 µM Cu and Fe typically accounts for ca. 5% of the cell protein. If this pMMO would have a cluster occupancy like the pMMO of
cells grown at high Cu and Fe, we would have seen in Figure 2A a doublet 1 with 1% resonance absorption. The observation that absorption attributable to doublet 1(if any) is less than 0.2 % suggests that the major fraction of pMMO in samples grown on low iron lacks the diiron cluster.

References


Table S1. Properties of whole cells and pMMO Mössbauer samples. pMMO activity is reported using as reductant either duroquinol (for purified pMMO) or formate (for whole cells).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Whole Cell</th>
<th>pMMO</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM pMMO</td>
<td>0.56*</td>
<td>1.06</td>
</tr>
<tr>
<td>Activity(^a)</td>
<td>302</td>
<td>157</td>
</tr>
<tr>
<td>Cu/(\alpha\beta\gamma)</td>
<td>-</td>
<td>7.3</td>
</tr>
<tr>
<td>Fe/(\alpha\beta\gamma)</td>
<td>-</td>
<td>1.1</td>
</tr>
<tr>
<td>Fe/sample (mM)</td>
<td>5.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Cu/sample (mM)</td>
<td>9.0</td>
<td>7.9</td>
</tr>
</tbody>
</table>

*Approximation based on pMMO comprising 20% total cell protein in cells cultured in media containing 80µM Cu.

\(^a\) nmol propylene oxidized \cdot min\(^{-1}\) \cdot mg total protein\(^{-1}\).
Figure S1. Top: SDS-denaturing gel of molecular mass standards (A), whole cell sample of *M. capsulatus* Bath cultured in NMS media supplemented with 80 µM CuSO₄ and 40 µM Fe⁵⁷ (B), washed membrane fraction of *M. capsulatus* Bath cultured in NMS media supplemented with 80 µM CuSO₄ and 40 µM Fe⁵⁷ (C), and of isolated pMMO (D). Samples were heated at 100°C for 30 s before loading before. Bottom: SDS-denaturing gel of whole cell (A), washed membrane fraction (B), and isolated pMMO. Samples were incubated at room temperature for 5 m before loading.

Figure S2. UV-visible absorption spectra of a 45 µM pMMO sample (aliquot of the Mössbauer sample of Figure 1). The band near 420 nm belongs to a contaminating cytochrome, either low-spin ferric cytochrome c₅₅₅ or possibly the species (1% of Fe) giving rise to the g = 6 EPR signal. No matter what the exact nature of this cytochrome might be, our sample cannot contain more than ca. 1% of the Fe bound to a heme.
Figure S3. X-band EPR spectra of *M. capsulatus* Bath cultured in NMS media supplemented with 80 µM CuSO$_4$ and 40 µM Fe$^{57}$ (1.06 mM αβγ) recorded at 25 K (A) and 11 K (B). Conditions: microwave frequency, 9.653 GHz; modulation amplitude, 1 mT at 100 kHz; microwave power, 20 µW (A) and 2 mW (B).
CHAPTER 8: GENERAL CONCLUSIONS

In methanotrophs, the initial oxidation of methane to methanol is catalyzed by methane monooxygenase (MMO). In some methanotrophs, two different MMOs can be expressed depending on the copper concentration during growth: a soluble cytoplasmic MMO (sMMO) in cells cultured under low copper/biomass and a membrane-associated, or particulate, MMO (pMMO) in cells cultured under high copper/biomass. While sMMO is well characterized enzyme that consists of a hydroxylase component composed of three polypeptides and a hydroxo-bridged binuclear iron cluster, a NADH-dependent reductase component composed of one polypeptide containing both FAD and [Fe₂S₂] cofactors, and a regulatory polypeptide (12, 25, 26, 34, 41).

Purification of the pMMO has been reported from *M. capsulatus* Bath (2, 24, 35, 43) and *M. trichosporium* OB3b (32, 39). The reporting laboratories agree that the pMMO is a copper-containing enzyme, composed of three polypeptides with molecular masses of approximately 45,000 (α-subunit), 26,000 (β-subunit), and 23,000 Da (γ-subunit) with a (αβγ)₂ molecular structure (4). However, researchers in the field disagree on the number and type of metal centers associated with the pMMO as well as the nature of the physiological electron donor.

**Metal centers of membrane associated methane monooxygenase.**

The crystal structure of pMMO refined to 2.8-A° resolution has been determined recently (23). The crystal structure showed that pMMO is a trimer, (αβγ)₃ with no iron
associated. Each αβγ monomer contained a binuclear copper center located near to membrane interface of α subunit, a mononuclear copper center found in the soluble region of α subunit above the membrane, and, surprisingly, a Zn occupied metal center located within the membrane of βγ complex (Fig. 1A-C). This study also proposed the binuclear copper center on α subunit as the catalytic center of pMMO.

The presence of Zn center of pMMO has never been predicted by any researchers in this field based on their spectroscopic studies. Instead, numbers of researchers have agreed on the presence of Fe center in pMMO (2, 24, 40, 43). Close examination of pMMO sample preparations for the crystal study (23) indicates the possibility of Zn displacement of Fe center. Sonication employed for cell lysis in this study are known to generate free radicals that can withdraw Fe from Fe containing proteins (10, 11, 14, 16, 20, 38). Considering this and high Zn concentration in their crystallization buffer (200mM zinc acetate), one can easily see Zn displacement of Fe center. In fact, our Mossbauer study (shown in chapter 7) with purified pMMO consistently indicates the presence of binuclear Fe cluster. When replaced with binuclear Fe cluster, this Zn occupied center shows striking similarity with the catalytic, binuclear iron center of sMMO hydroxylase component (Fig 1E, 2) (40). Furthermore, close examination of the deposited pMMO crystal structure revealed conserved hydrophilic amino acids within 10 Å radius from the Zn center (shown in chapter 7) that can provide additional ligation for binuclear Fe coordination (Fig 2A).

Lemos et. al. (22) observed the presence of type II Cu$^{2+}$ center from their EPR study with isotopically enriched pMMO expressing cells and predicted 3 histidine
imidazole N + 1 amine N coordination (Fig. 3). Interestingly, when binuclear Cu center of β subunit shown in the crystal structure is displaced with mononuclear Cu (Fig. 1D) the Cu coordination becomes almost identical to their prediction.

As discussed above, binuclear Cu center and Zn center shown in the crystal structure can be replaced with mononuclear Cu and binuclear Fe, respectively. Once replaced, binuclear Fe center becomes an attractive alternative for the catalytic center of pMMO due to its resemblance with the catalytic center of sMMO hydroxylase component. However, this possibility need to be further examined.

**Methanobactin: A moonlighting protein.**

Methanobactin (mb) is a small 1154 Da Cu-binding chromopeptide initially identified in the methanotroph *Methylococcus capsulatus* Bath. Mb appears to act as the intracellular component of a Cu acquisition system. When bound to Cu, mb (Cu-mb) also exhibits superoxide dismutase, reductant dependent-oxidase, and -hydrogen peroxide reductase activities. Reductant dependent-oxidase activity of mb can reduce molecular oxygen (O₂) to superoxide (O₂⁻) in the presence of atmospheric oxygen and also can directly reduce NBT to insoluble formazan in the absence of oxygen. Numbers of studies have shown O₂⁻ is formed by accident when molecular oxygen adventitiously withdraws an electron from redox components of respiratory chain that transfer electrons to other substrates (5-7, 28-30). In fact, none of the enzymes that have been characterized are thought to generate O₂⁻ as a deliberate, stoichiometric product. Studies have also shown these redox active components were able to directly reduce NBT to insoluble formazan in the absence of oxygen (19, 36, 37) like mb indicating mb might also be a redox
component of the respiratory chain of methanotrophs. This concept is consistent with the results presented in this thesis showing the addition of Cu-mb enhanced pMMO activity in washed membrane fractions.

Inhibitory effect of mb from *Ms. trichsporium* OB3b on pMMO activity at low Cu to mb ratios can be explained by the generation of $O_2^-$ by mb. In *Escherichia coli* (11, 15-17, 31), when respiratory vesicles were incubated in vitro with reductant and atmospheric oxygen, normal electron transport was accompanied by the generation of $O_2^-$ which in turn caused further damages on variety of biomolecules. Considering mb does not show any detectable SOD-like or reductant-dependent HPR activities at low Cu to mb ratios, the inhibitory effects of mb on pMMO activity was probably due to the increased reactive oxygen radical generations by added mb.

In conclusion, mb may be the redox active component of respiratory chain directly linked to pMMO (Fig. 4) with radical scavenging activities such as SOD-like activity, as well as reductant dependent HPR activity. These radical scavenging activities of mb may play an additional important role protecting the biological molecules including pMMO from radical-mediated damage. The multiple function of mb suggests mb may belong to a noble growing group of protein known as “moonlighting proteins” (3, 18, 33).

**Methanobactin: Other properties.**

Methanobactin (mb) binds to a variety of metals. The binding of different metals by mb is intriguing and suggests that although mb preferentially binds copper, mb produced by methanotrophs may play a role in mobilization/immobilization of may
metals *in situ*. One of the persistent and substantial problems in remediation of hazardous waste site is the mobilization and transport of radionuclides and heavy metals from these sites to surrounding areas (1, 9, 21). Because methanotrophs are often present at these sites, the ability of mb binding to different metals suggests methanotrophs may be responsible or involved in the mobilization of radionuclides and heavy metals.

Mb also binds to Au(III), Pd(II), Pt(II), and Ru(II) and reduces to elemental states producing nanocystalline structures such as nanoparticles and/or nanosheets in the absence of reductant (data not shown). Because the reactivity of catalyst increases dramatically at nanoscale—called nanocatalysts—and nanocatalysts often exhibits noble reactivity different from the bulk material of the same composition (8, 27, 42), this nanostructure generating property of mb may be useful to develop nanocatalysts.

**References**


Figure 1. Metals centers of pMMO. (A) binuclear copper center of α subunit. (B) mononuclear copper center of α subunit. (C) mononuclear zinc center of βγ subunit complex. (D) binuclear copper center of α subunit replaced by mononuclear copper. (E) mononuclear zinc center of βγ subunit complex replaced by binuclear iron. Modified from ref. 23. ■: Nitrogen, ■: Oxygen.
Figure 2. A. Replacement of the mononuclear Zn by a binuclear iron center in the X-ray structure of pMMO (23). B. Binuclear iron center of sMMO hydroxylase component (extracted from PDB entry 1MTY).


Figure 3. Schematic drawing for the proposed binding sites for typeII Cu$^{2+}$ in pMMO. Adopted from ref. 22.
Figure 4. Proposed pathways of methane oxidation in *Methylococcus capsulatus* Bath. Cu-mb may be the redox active component of respiratory chain directly linked to pMMO.