1378 *Inorg. Chem.* **2011**, *50*, 1378–1391
DOI: 10.1021/ic101965j

Inorganic Chemistry
Article

Copper-Binding Properties and Structures of Methanobactins from *Methylosinus trichosporium* OB3b

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Received September 25, 2010

Methanobactins (mbs) are a class of copper-binding peptides produced by aerobic methane oxidizing bacteria (methanotrophs) that have been linked to the substantial copper needs of these environmentally important microorganisms. The only characterized mbs are those from *Methylosinus trichosporium* OB3b and *Methylocystis* strain SB2. *M. trichosporium* OB3b produces a second mb (mb-Met), which is missing the C-terminal Met residue from the full-length form (FL-mb). The as-isolated copper-loaded mbs bind Cu(I). The absence of the Met has little influence on the structure of the Cu(I) site, and both molecules mediate switchover from the soluble iron methane mono-oxygenase to the particulate copper-containing enzyme in *M. trichosporium* OB3b cells. Cu(II) is reduced in the presence of the mbs under our experimental conditions, and the disulfide plays no role in this process. The Cu(I) affinities of these molecules are extremely high with values of $(6-7) \times 10^{20}$ M^{-1} determined at pH $\geq$ 8.0. The affinity for Cu(I) is 1 order of magnitude lower at pH 6.0. The reduction potentials of copper-loaded FL-mb and mb-Met are 640 and 590 mV respectively, highlighting the strong preference for Cu(I) and indicating different Cu(II) affinities for the two forms. Cleavage of the disulfide bridge results in a decrease in the Cu(I) affinity to $\sim 9 \times 10^{18}$ M^{-1} at pH 7.5. The two thiolates can also bind Cu(I), albeit with much lower affinity ($\sim 3 \times 10^{15}$ M^{-1} at pH 7.5). The high affinity of mbs for Cu(I) is consistent with a physiological role in copper uptake and protection.

Introduction

Most microbes need to acquire copper and iron from their surroundings. Many organisms produce siderophores, small molecules that solubilize and take up Fe(III), and these have been thoroughly investigated.[1] However, analogous molecules involved in copper acquisition are less well characterized. Some aerobic methane-oxidizing bacteria (methanotrophs) secrete small copper-binding molecules called methanobactins (mbs).[2,3] Almost all methanotrophs initiate methane utilization, and consequently the suppression of this greenhouse gas, through the action of a copper-containing particulate methane mono-oxygenase (pMMO).[4,5] However, some methanotrophs alternately express a soluble MMO (sMMO) under low copper conditions[6−8] that has a dinuclear iron active site.[9] There has been considerable controversy about the active site of pMMO, but recent work indicates that a dinuclear copper center is the site of methane oxidation in the enzyme from *Methylococcus capsulatus* Bath,[10] which is probably also the case in other species. Regardless of the methanotroph, copper requirements are conditionally high because of their copper-dependent pMMO, and studies on *Methylosinus trichosporium* OB3b, a methanotroph that produces both sMMO and pMMO, show that mb facilitates copper uptake.[11] The exact regulatory mechanism of

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 Published on Web 01/21/2011

switchover between sMMO and pMMO is not fully understood, but mb appears to mediate the process,[12,13] particularly in the presence of mineral copper sources,[14] and is implicated in other key functions in these environmentally important organisms.[3,5]

Mbs are thought to be present in many methanotrophs,[15−17] but the molecules from *M. trichosporium* OB3b[2] and *Methylocystis* strain SB2[18] are the only two that have been characterized. Both of these are modified peptides, and that from *M. trichosporium* OB3b, which has been studied in the greatest detail, has the sequence 1-(N-[mercapto-{5-oxo-2-(3-methylbutanoyl)oxazol-(Z)-4-ylidene}methyl]-Gly[1]-L-Ser[2]-L-Cys[3]-L-Tyr[4])-pyrrolidin-2-yl-(mercapto-[5-oxo-oxazol-(Z)-4-ylidene]methyl)-L-Ser[5]-Cys[6]-L-Met[7] (Figure S1, Supporting Information).[19] The two modified amino acids in this mb were first believed to be hydroxyimidazolate rings,[2] but alkylidene oxazolone rings are now favored.[19] The recently characterized mb from *Methylocystis* strain SB2 is significantly shorter, containing three Ala residues and a Ser along with the two modified residues, providing single oxazolone and imidazolone rings (Figure S1, Supporting Information).[18] The crystal structure of *M. trichosporium* OB3b mb reveals a pyramid-like shape with a disulfide bond between the two Cys residues.[2] A single copper ion is bound at the base of the pyramid by a N_2S_2 ligand set derived from the modified amino acids. *M. trichosporium* OB3b and *Methylocystis* strain SB2 mbs bind both Cu(II) and Cu(I), with Cu(II) reduced upon uptake.[18,20−23]

In this work, we have fully characterized two distinct mbs from *M. trichosporium* OB3b. Both molecules bind copper in an identical arrangement, as shown by high-resolution crystal structures, and can function *in vivo* in a similar manner. We have measured the Cu(I) affinities of the mbs, and from the determination of their reduction potentials (E_m values), Cu(II) affinities have been calculated. We also demonstrate the effect of pH and disulfide bond cleavage on Cu(I) affinity. The high copper affinity of mb indicates that copper uptake and protection are its primary biological functions. These studies also show that methanotrophs can produce modified forms of the same copper-binding molecule, as is the case for certain peptidic siderophores.[24]

Experimental Section

***M. trichosporium* OB3b mb Production and Purification.** A modified version of a published procedure[20] was used to obtain mb from spent media of *M. trichosporium* OB3b cultures. Cells were grown at 27 °C in a 5 L fermentor in a nitrate minimal salts (NMS) medium[25] supplemented with 1 μM copper. The material eluted from C_{18} cartridges (Sep-Pak Plus, Waters) with 60% acetonitrile was lyophilized overnight and then resuspended in 10 mM ammonium acetate at pH 6.5 containing 10 mM ethylenediaminetetraacetic acid (EDTA) and passed through a 0.45 μm filter. Reverse phase (RP) high performance liquid chromatography (HPLC) was carried out on a semi-preparative ACE 10 column (C_{18}-300, 250 × 10 mm, Hichrom) attached to an Agilent 1100 Series instrument equipped with a diode array detector at a flow rate of 2 mL/min. The buffers used were 10 mM ammonium acetate at pH 6.5 (buffer A) and 10 mM ammonium acetate at pH 6.5 in 80% acetonitrile (buffer B). A linear gradient from 5% to 45% buffer B over 40 min was used to purify the mbs. If EDTA is omitted, mbs elute at higher volumes with other metal ions bound, including zinc and nickel. Pure mb fractions were lyophilized overnight and stored at −20 °C. In order to check the purity of collected samples, high-resolution HPLC was performed on an analytical ACE 3 column (C18−300, 150 × 2.1 mm, Hichrom) at a flow rate of 0.2 mL/min. Samples were treated with 10 equivalents of EDTA, and a linear 25 min gradient from 15% to 40% buffer B was used.

Stability of mb. Two distinct mbs are isolated from the spent media of *M. trichosporium* OB3b cultures, one of which corresponds to the previously characterized[2] full length molecule (FL-mb), and a shortened form. The stability of apo-FL-mb and Cu(I)-FL-mb were determined by adding each separately (both at 0.25 μM) to 400 mL of cell-free spent media recovered from a culture grown in the presence of 1 μM copper and harvested at an optical density of 1.2 at 600 nm. These solutions were incubated at 27 °C with shaking (120 rpm). Samples (100 mL) were removed immediately after the addition of mb and then after 24, 48, and 96 h, loaded onto C_{18} cartridges, eluted, lyophilized, and analyzed by preparative HPLC as described above.

Mass Spectrometry. As-isolated mbs [apo and Cu(I) forms] and also apo-mbs loaded with either Cu(I) or Cu(II) (> 1 equivalent, *vide infra*) were desalted and concentrated using RP-C_{18} StageTips (Thermo Scientific) prior to electrospray ionization mass spectrometry (ESI-MS). The mbs were loaded onto tips, washed three times with 20 mM ammonium acetate at pH 6.5 (10 μL), and then eluted with 50% acetonitrile plus 5% ammonium hydroxide (3 μL) into medium NanoES spray capillaries (Thermo Scientific). Electrospray analysis was performed in negative ion mode on a Finnigan LTQ-Fourier transform (FT) mass spectrometer (Thermo Scientific). Data were acquired with a typical FT-MS resolution setting of 100 000 (at m/z = 400), and MS spectra were analyzed using the QualBrowser program (Thermo Scientific). The accuracy of mass data is significantly better than 0.05 Da.

Copper Concentration Determinations. The copper concentration of purified samples was determined by atomic absorption spectrometry (AAS, M Series, Thermo Scientific). Copper

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Table 1. Data Collection and Processing Statistics

	Cu(I)-FL-mb	Cu(I)-mb-Met	Cu(I)-FL-mb	Cu(I)-mb-Met
beamline	ESRF BM 14.4	ESRF BM 14.4	Diamond IO4	Diamond IO4
		data statistics[a]		
wavelength (Å)	0.9185	0.97848	0.91	0.91
resolution (Å)	20.79−1.10 (1.16−1.10)	21.37−1.15 (1.21−1.15)	20.84−0.92 (0.97−0.92)	21.14−1.00 (1.05−1.00)
space group	$P4_12_12$	$I222$	$P4_12_12$	$I222$
unit-cell parameters: a, b, c (Å)	29.40, 29.40, 29.40	31.69, 39.53 84.97	29.48, 29.48, 29.48	31.71, 39.57, 85.05
$\alpha = \beta = \gamma$ (deg)	90	90	90	90
unit-cell volume (Å^3)	25412.2	106442.4	25611.5	106717.7
solvent content (%)	40.0	64.9	40.0	64.9
no. of measured reflns	30868	125793	102587	198467
no. of independent reflns	5550	18308	9025	29329
completeness (%)	98.7 (98.9)	94.5 (69.3)	95.7 (78.8)	99.8 (100.0)
redundancy	5.6 (3.2)	6.9 (3.5)	11.3 (5.5)	6.8 (6.4)
Rmerge (%)	12.3 (31.8)	5.4 (30.3)	6.2 (33.5)	9.0 (36.5)
$<I>/<\sigma(I)>$	7.3 (2.7)	9.4 (2.5)	25.1 (5.5)	13.5 (4.4)
		refinement statistics		
R_{work} (%)			12.45	11.37
R_{free}[b] (%)			14.05	12.91
no. of protein atoms			146	438
no. of solvent atoms			13	58
no. of Cu atoms			2	6
RMS deviation from ideal values				
bond length (Å)			0.020	0.026
angle distance (Å)			0.051	0.062
average B factor (Å^2)				
protein			8.28	9.00
Cu			5.57	7.05
solvent			17.77	20.70
Ramachandran plot,[c] residues in most favored regions (%)			100	100

[a] Values in parentheses are for the highest resolution shell. [b] 5% test set in thin shells. [c] Calculated using MOLPROBITY.

standards (concentrations in the 0.2−1 ppm range) were made from a copper stock solution of 987 μg/mL in 2% HNO_3 (Sigma-Aldrich). The mb samples were incubated in 2% HNO_3 prior to analysis.

Crystallization and Structure Determinations. Initial crystal trials were carried out at 20 °C with commercial screens using the sitting drop method of vapor diffusion in 96-well plates set up with a crystallization robot mixing 0.1 and 0.2 μL of Cu(I)-mb (12−15 mg/mL) in 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes) at pH 7.5 with 0.1 μL of the reservoir solution. Conditions were optimized using the hanging drop method of vapor diffusion at 20 °C. All drops were generated by mixing 2 μL Cu(I)-mb (12−15 mg/mL) in 20 mM Hepes at pH 7.5 with 1 μL of the precipitant solution and equilibrated against 0.5 mL of the precipitant solution. Crystals suitable for X-ray diffraction studies were obtained from 4 M sodium formate for FL-mb and from 2 M ammonium sulfate plus 5% (v/v) isopropanol for the shortened form. All crystals were frozen in *N*-paratone oil except for the highest resolution data for the new form of mb, which was frozen directly in liquid nitrogen.

X-Ray Data Collection and Structure Refinement. Initial diffraction data for both forms of mb were collected at beamline ID 14.4 of the ESRF to ∼1.1 Å, allowing structure solution. Higher resolution data, to 0.92 and 1.00 Å (Table 1), were subsequently collected on beamline IO2 at the DLS. All data were processed and integrated using iMOSFLM[26] and scaled using SCALA.[27] Crystal structures were solved by single-wavelength anomalous dispersion (SAD) using the anomalous signal of the copper ions. At the collection wavelength of 0.9185 Å [for Cu(I)-FL-mb], $f'' = 1.9$ e and is approximately half the value at the peak ($f'' = 3.9$ e). However, mb is relatively small, ensuring that there was a more than sufficient anomalous signal for structure solution by SAD. The sites were determined using SHELXD, and the phases were obtained using SHELXE on the data sets collected at the ESRF.[28] The previously reported mb structure (Cambridge Crystallographic Data Centre, deposition number CCDC 241254)[2] was manually fitted into the electron-density map obtained from SHELXE using COOT[29] and used as a starting model for structure building and refinement. The models were completed using iterative cycles of refinement with SHELXL and model-building using COOT.[29] A total of 5% of reflections, selected in thin shells, were assigned as the R_{free} set and excluded from refinement. This set was extended to higher resolution for the data from DLS. The final cycles of model refinement were performed against these data with temperature factors refined anisotropically and hydrogens added in riding positions. The models were validated using Molprobity.[30] The atomic coordinates for both Cu(I)-FL-mb and Cu(I)-mb-Met have been deposited in the Protein Data Bank (PDB) with the accession codes 2xjh and 2xji, respectively.

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***In Vitro* Analysis of mbs.** For metal binding studies, the mb samples were routinely in 20 mM Hepes at pH 7.5. Additions of Cu(II) were made from a $CuSO_4 \cdot 5H_2O$ stock solution (100 mM) prepared in deionized water and serially diluted in 20 mM Hepes at pH 7.5. For Zn(II) titrations, a stock solution (100 mM) of $ZnSO_4 \cdot 7H_2O$ was prepared in 20 mM Hepes at pH 7.5. A stock solution (25−50 mM) of tetrakis(acetonitrile)copper(I)-hexafluorophosphate ($[Cu(CH_3CN)_4]PF_6$) prepared in an anaerobic chamber (Belle Technology) by dissolving the salt quantitatively in deoxygenated acetonitrile and serially diluting in 20 mM Hepes at pH 7.5 was used for additions of Cu(I). Copper and zinc concentrations were checked by AAS. For UV−visible (UV−vis) spectra (all collected at 25 °C on a PerkinElmer λ35 spectrophotometer), samples were transferred to an anaerobic quartz cuvette (1 cm path-length, Hellma) and sealed with a septum cap in an anaerobic chamber for experiments under oxygen-free conditions. Titrations were made using an airtight syringe equipped with a PB600 repeating dispenser (Hamilton). UV−vis spectra were recorded after waiting for the absorbance at 340 (copper) or 394 (zinc) nm to be stable [typically ~10 min for Cu(II) but < 1 min for Cu(I) and Zn(II) additions]. At the end of titrations in which up to 2 equivalents of Cu(II) had been added, 1 equivalent of EDTA was added to the sample and a UV−vis spectrum measured. To monitor the reduction of Cu(II)-mbs, UV−vis spectra were collected at ~10 s intervals after the addition of Cu(II) (9.5 μM) to copper-free (apo) peptide (10 μM). The influence of pH on both apo- and Cu(I)-mbs was monitored by comparing a UV−vis spectrum in 20 mM Hepes at pH 7.5 to those left in 20 mM sodium acetate (at pH 5.5, 4.5, 3.8, and 2.1) for approximately 20 h.

The addition of Cu(I) to the apo-mbs in the presence of bicinchoninic acid (BCA, $\log(\beta_2) = 17.2$ at pH 7[31]) was monitored by UV−vis to determine the concentrations of the apo-mbs. The $[Cu(BCA)_2]^{3-}$ complex absorbs strongly at 562 nm, and a molar extinction coefficient of 7.8 $mM^{-1}\ cm^{-1}$ in 20 mM Hepes at pH 7.5 has been determined from Cu(I) titrations into an excess of BCA, which is in good agreement with the literature value of 7.9 $mM^{-1}\ cm^{-1}$.[31] The absorbance at 562 nm was corrected for the contribution from Cu(I)-mbs ($\varepsilon_{Cu(I)\text{-}mb} = 0.4\ mM^{-1}cm^{-1}$).

EPR Spectroscopy. EPR spectra were acquired of the as-isolated copper-loaded mbs (2 mM) in 20 mM Hepes at pH 7.5. Spectra were also acquired of apo-mbs (1−2 mM) incubated with 0.3−1.4 equivalents of Cu(II) for >10 min at room temperature in 20 mM Hepes at pH 7.5 prior to freezing in liquid nitrogen. EPR was used to analyze a sample of apo-mb-Met (1 mM) incubated with Cu(II) (800 μM) for 10 s and 1, 2, 5, and 10 min prior to freezing. All spectra (20 K) were recorded at X-band on a Bruker EMX spectrometer equipped with an ESR900 continuous flow cryostat (Oxford Instruments). The microwave frequency was 9.41 GHZ, the microwave power 10 mW, the field modulation 5 G, and the time constant and the conversion time were both 40.96 ms. Spectra were processed and simulated using WIN EPR and SIMFONIA (Bruker), respectively.

Reduction of Apo-mbs and Thiol Quantification. Apo-mbs were reduced using immobilized tris(2-carboxyethyl)phosphine (TCEP) gel (Thermo Scientific) under anaerobic conditions. One hundred μL of apo-mb (~ 1 mM) was mixed for 45 min with 200 μL of the TCEP gel (initial concentration of TCEP ~ 8 mM), washed three times with 20 mM Hepes at pH 7.5 prior to incubation. The gel was removed using two rounds of centrifugation at 2000 rpm followed by passing the supernatant through a 0.2 μm filter. The presence of free thiols was assessed by the reaction of 10−20 μM apo-mb with 200 μM Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid), DTNB)[32,33] in 100 mM potassium phosphate at pH 8.0 plus 1 mM EDTA. A molar extinction coefficient of 14 150 $M^{-1}\ cm^{-1}$ at 412 nm was used for the 5-thio-2-nitrobenzoate ion product.[33]

Cu(I) Affinity Determinations. Cu(I) affinities were typically determined by competition experiments with the chromophoric Cu(I) chelator bathocuproine disulphonate (BCS) in 20 mM Hepes at pH 7.5 and 25 °C. The $[Cu(BCS)_2]^{3-}$ complex exhibits an absorption maximum at 483 nm, and a molar extinction coefficient of 12 800 $M^{-1}cm^{-1}$ has been determined from Cu(I) titrations into an excess of BCS in 20 mM Hepes at pH 7.5 (this is slightly lower than the literature value[34] of 13 300 $M^{-1}\ cm^{-1}$). The overall stability constant β_2^{max} for $[Cu(BCS)_2]^{3-}$ is $10^{19.8}\ M^{-2}$, and the β_2 value at different pH values has been calculated from eq 1 using a pK_a of 5.7 for BCS:[34]

$$\beta_2 = \frac{\beta_2^{max}}{(1+[H^+]/K_a)^2} \quad (1)$$

Titrations of apo-mb (0.5−1 mM) into ~10 μM $[Cu(BCS)_2]^{3-}$ required a large excess of BCS (up to 5 mM) to be added to promote effective competition. To ensure equilibrium conditions had been achieved, reverse titrations were also performed in which BCS was added to Cu(I)-mb. Titrations in this direction were also carried out at different pH values in the 6.0 to 10.5 range in the presence of 200 mM NaCl using 20 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes) at pH 6 and 6.5, 20 mM Hepes at pH 7.0 and 7.5, 20 mM N-tris-(hydroxymethyl)methyl-3-aminopropanesulfonic acid (Taps) at pH 8.5, 20 mM 2-(N-cyclohexylamino)ethane sulfonic acid (Ches) at pH 9.5, and 20 mM *N*-cyclohexyl-3-aminopropanesulfonic acid (Caps) at pH 10.5 as buffers. After the final addition of BCS, the pH value was checked to ensure that no change had occurred (typically less than 0.1 pH unit). The Cu(I) affinity of an apo-mb sample loaded with 0.6−0.7 equivalents of Cu(II), with the copper allowed to reduce fully, was also determined from a titration with BCS at pH 7.5. The Cu(I) affinity (K_b) of mb can be expressed as follows:[34,35]

$$K_b = \frac{([Cu]_t - [Cu(BCS)]_2)([BCS]_t - 2[Cu(BCS)_2])^2\beta_2}{[Cu(BCS)_2]([mb]_t + [Cu(BCS)_2] - [Cu]_t)} \quad (2)$$

where $[Cu]_t$ is the total concentration of Cu(I), $[mb]_t$ is the total concentration of mb, $[BCS]_t$ is the total concentration of BCS, and $[Cu(BCS)_2]$ is the concentration of the $[Cu(BCS)_2]^{3-}$ complex in solution. $[Cu(BCS)_2]$ was calculated from the absorbance at 483 nm (A_{483nm}) corrected for the absorbance from Cu(I)-mbs ($\varepsilon_{Cu(I)\text{-}mb} = 2.5\ mM^{-1}\ cm^{-1}$) according to eq 3:

$$[Cu(BCS)_2] = \frac{A_{483nm} - \varepsilon_{Cu(I)-mb}[Cu]_t}{\varepsilon_{[Cu(BCS)_2]} - \varepsilon_{Cu(I)-mb}} \quad (3)$$

Experimental data were fit to eq 2 using Origin 7.0 to obtain K_b values. The pH dependence of K_b for Cu(I)-mb-Met could be fit to eq 4:

$$\log(K_b) = \log(K_b^{max}) - \log(1 + 10^{(pK_a - pH)}) \quad (4)$$

allowing the pK_a of the ionizing group that influences the Cu(I) affinity in the pH range studied, and the affinity for the fully deprotonated form (K_b^{max}), to be determined.

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(34) Xiao, Z. G.; Loughlin, F.; George, G. N.; Howlett, G. J.; Wedd, A. G. *J. Am. Chem. Soc.* **2004**, *126*, 3081–3090.
(35) Xiao, Z.; Wedd, A. G. *Nat. Prod. Rep* **2010**, *27*, 768–789.

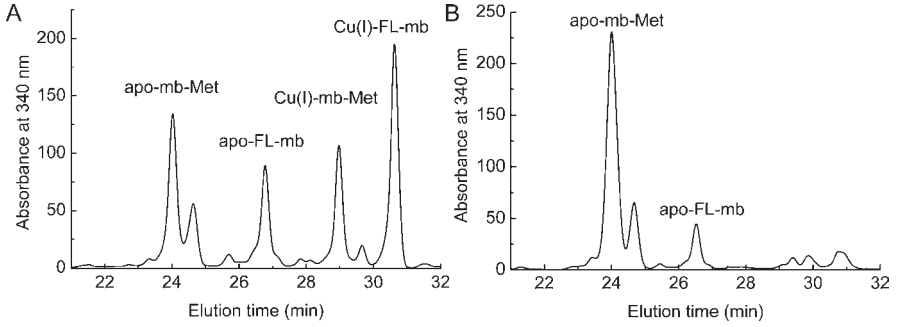

Figure 1. Semi-preparative HPLC chromatograms of *M. trichosporium* OB3b extracts. In A, a mixture of both mbs is obtained, whereas in B, the shortened form is the major mb present in the extract.

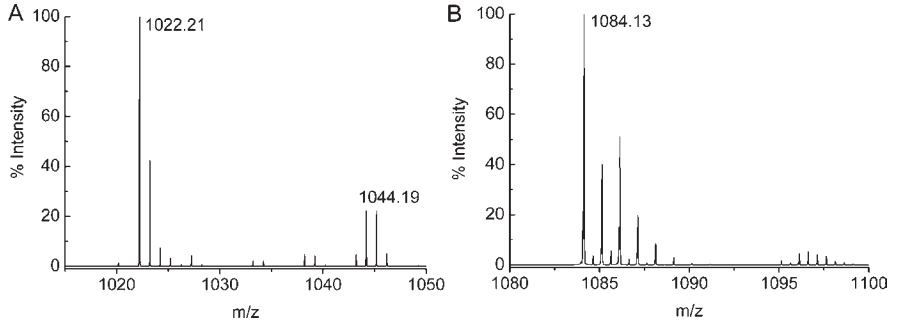

Figure 2. ESI-MS analysis of apo-mb-Met (A) and Cu(I)-mb-Met (B). In A, the peak at 1044.19 Da is the sodium adduct of the main signal.

To independently check the relative copper affinities of the two mbs, a mixture of the apo forms (both 50 μM) plus 50 μM Cu(II) was incubated in 20 mM Hepes at pH 7.5. Ten equivalents of EDTA were added, and the mixture was analyzed by analytical HPLC as described above. The transfer of metal between the Cu(I) form of one mb and the apo form of the other was also analyzed using this approach in 20 mM Hepes at pH 7.5.

The mbs in which the disulfide had been reduced by TCEP bind Cu(I) tightly via the same site as the peptides in which the disulfide is intact, and the affinity of this site was determined as described above. The disulfide-reduced forms of the mbs also bind Cu(I) via the two thiols, and the affinity of this weaker site was determined from competition experiments with BCA. Experiments were performed by titrating BCA into reduced mbs loaded with ~1.6 equivalents of Cu(I), and data were analyzed in the same way as for experiments with BCS, except that the $\varepsilon_{Cu(I)\text{-}mb}$ of 0.4 mM^{-1} cm^{-1} at 562 nm was used.

Electrochemistry. The direct measurement of the E_m values of the copper-loaded mbs (~500 μM) was carried out by cyclic voltammetry using a gold working electrode and a setup described previously.[36] The gold electrode was modified by immersion in a saturated solution of diphenyldisulfide for 5−10 min. Measurements (at 25 ± 1 °C) were typically performed at 20 mV/s in 20 mM tris(hydroxymethyl)aminomethane (Tris) at pH 7.0 plus 90 mM NaCl and 20 mM Hepes at pH 7.5 plus 90 mM NaCl. All E_m values are quoted referenced to the normal hydrogen electrode (NHE) and were calibrated using the $[Co(phen)_3]^{3+/2+}$ couple (370 mV vs NHE).

Influence of mbs on sMMO and pMMO Expression in *M. trichosporium* OB3b. *M. trichosporium* OB3b cells were grown in NMS media without copper to an optical density of 1.0 at 600 nm (~430 mg/L cell dry weight). sMMO activity was verified using the *o*-dianisidine/naphthalene spectrophotometric assay.[12,37] Thirty milliliters of culture was collected, centrifuged at 14 000*g* for 5 min, and washed twice with 1× phosphate-buffered saline solution to remove residual mb. The cells were resuspended in fresh NMS media and redistributed (5 mL each) into sealed 30 mL serum vials. Methane (30%) was provided, and the cultures were allowed to recover for 1 h. As a pretreatment control, 300 μL were removed from each vial using a TB syringe and were rapidly preserved in 1 mL QiaZol reagent and immediately frozen at −80 °C. At time 0, apo- and copper-bound mbs and $CuCl_2$ (final concentration 2 μM in all cases) were added to different replicate vials (a control vial which contained cells only was also included). After 10 min (time chosen on the basis of previous studies[14]), 300 μL of culture was removed from each vial, preserved in 1 mL QiaZol reagent, and frozen at −80 °C. mRNA was extracted using an RNeasy MinElute Cleanup kit (Qiagen). Multiplex reverse-transcriptase qPCR was performed on the freshly extracted samples based on previously published methods[14] using a BioRad iCycler thermal cycler (Bio-Rad). Two microliters of mRNA template was added to prepared iScript Supermix (Bio-Rad) reagent solution with 15 pmol of primers and 6.26 pmol Taqman probes simultaneously targeting *pmoA* and *mmoX* transcripts. These targets were chosen because *pmoA* codes for the β-subunit of pMMO, reflecting pMMO manufacture, whereas *mmoX* codes for the α-hydroxylase subunit of sMMO and reports on the manufacture of the alternate MMO.[14] Transcript levels are reported as a ratio to minimize the influence of small differences in reaction and extraction efficiencies between assays.

Results

Isolation of mbs from *M. trichosporium* OB3b. *M. trichosporium* OB3b cultures produce two different mbs that can be separated on a semi-preparative HPLC column (Figure 1 and Table 2). The purity of these fractions was verified by analytical HPLC (Figure S2,

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Table 2. Elution Times of apo- and Cu(I)-mbs Obtained by Semi-Preparative and Analytical HPLC and the Molecular Weights Determined by ESI-MS

	elution times (min)					
	semi-preparative column		analytical column		molecular weight (Da)[a]	
mb	apo	Cu(I)	apo	Cu(I)	apo	Cu(I)
mb-Met	24	29	10.1	14.6	1022.21	1084.13
FL-mb	26.5	30.5	11.6	15.3	1153.24	1215.18

[a] Apo mass = $[M - H]^-$, Cu(I)-mb = $[M + Cu - 2H]^-$, and the mass difference = [Cu − H] = 62.93 − 1.01 = 61.92.

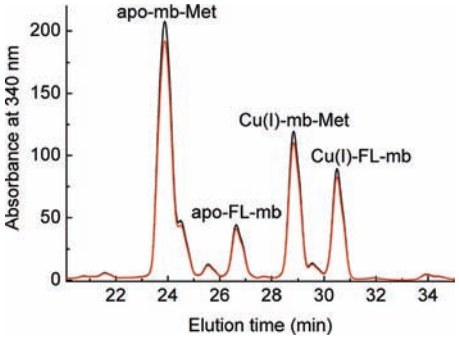

Figure 3. Semi-preparative HPLC chromatograms of cell-free spent media to which Cu(I)-FL-mb (0.25 μM) was added immediately prior to mb isolation and analysis (black line) and that for the same mixture incubated for 96 h at 27 °C (red line).

Supporting Information). ESI-MS (Figure 2 and Table 2 and Figure S3 in the Supporting Information) identifies these as FL-mb and a form that is 131 Da smaller (both are present with and without copper in Figure 1A). This mass difference is indicative of the loss of the Met residue at the C-terminus (mb-Met, see Figure S1 in the Supporting Information). FL-mb and mb-Met are routinely found in *M. trichosporium* OB3b cultures grown under a variety of different conditions (including with and without 1−2 μM Cu and elevated versus low O_2 and/or methane levels), and on some occasions mb-Met is the major form obtained from a culture (Figure 1B). Efforts are ongoing to identify consistent growth conditions for the production of FL-mb versus mb-Met, but they have not yet been identified. *In vitro* removal of the C-terminal Met of FL-mb has previously been achieved using proteases,[20] and the stability of FL-mb in cell-free spent media has therefore been tested. After incubation for 96 h, there is no indication that FL-mb is converted to mb-Met by possible proteases in the spent media (Figure 3), and mb-Met is produced by *M. trichosporium* OB3b.

Crystal Structures of mbs. To confirm the absence of the C-terminal Met residue, and to determine the effect of this modification on the overall structure, we crystallized Cu(I)-mb-Met (Figure 4A, Table 1, and Figure S4A in the Supporting Information). We were also able to crystallize the Cu(I)-bound form of FL-mb (Figure 4B, Table 1, and Figure S4B in the Supporting Information) under very different conditions from those previously reported[2] and obtained a structure at sub-Ångström resolution. The same space group and an almost identical overall structure [root-mean-square deviation (RMSD) of 0.08 Å from an overlay of the C^α atoms of the seven standard amino acids] are found for Cu(I)-FL-mb. In the Cu(I)-mb-Met structure, two of the six molecules in the asymmetric unit exhibit significant mobility, and it was necessary to

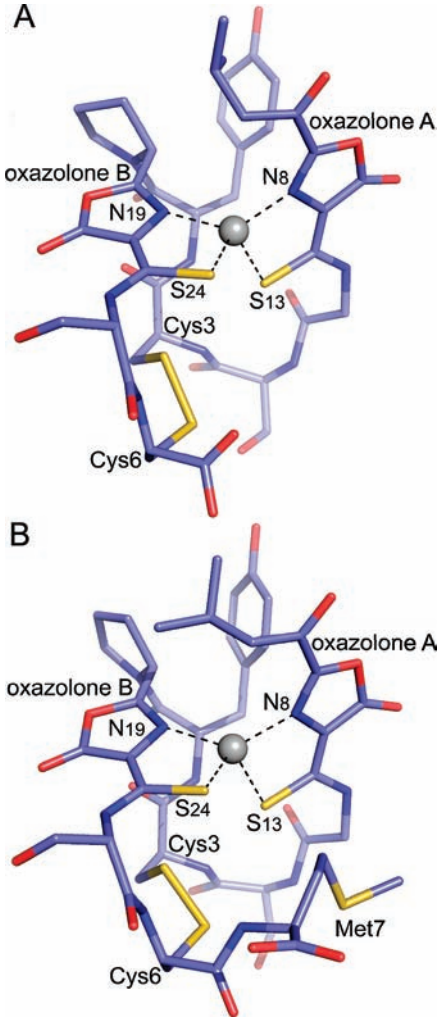

Figure 4. Structures of Cu(I)-mb-Met (A) and Cu(I)-FL-mb (B) with the copper ions represented as silver spheres.

model a second position (with 30% occupancy) for the peptide and the copper ion for these. The mobility is likely due to the very open packing arrangement in these crystals, particularly adjacent to the two more mobile molecules. Our structural data for Cu(I)-mb-Met and Cu(I)-FL-mb are consistent with the presence of oxazolone rings, and with the N-terminus being an isobutyl[19] rather than an isopropylester[2] group.

The overall structures of Cu(I)-mb-Met and Cu(I)-FL-mb (Figure 4) are similar (RMSD of 0.25 Å for an overlay of the C^α atoms of the six standard amino acids). This is as expected considering the Met makes a limited number of interactions in FL-mb (Figure 4B). The loss of the Met results in one of the Cys residues being at the C-terminus, but this has no effect on the disulfide bridge. In both

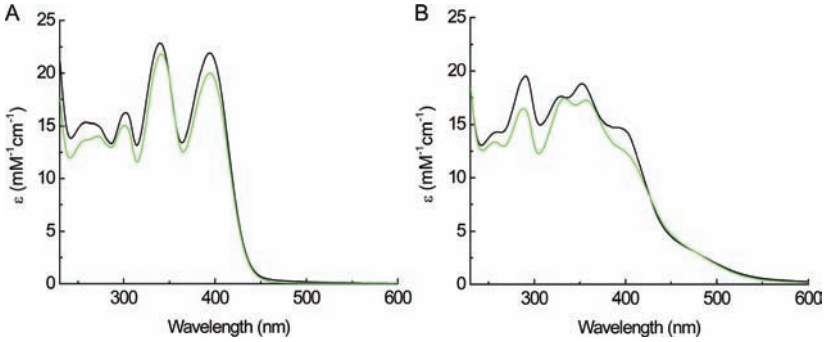

Figure 5. UV−vis spectra (25 °C) of apo- (A) and as-isolated Cu(I)-mbs (B) in 20 mM Hepes at pH 7.5. The spectra of mb-Met and FL-mb are shown in black and green, respectively.

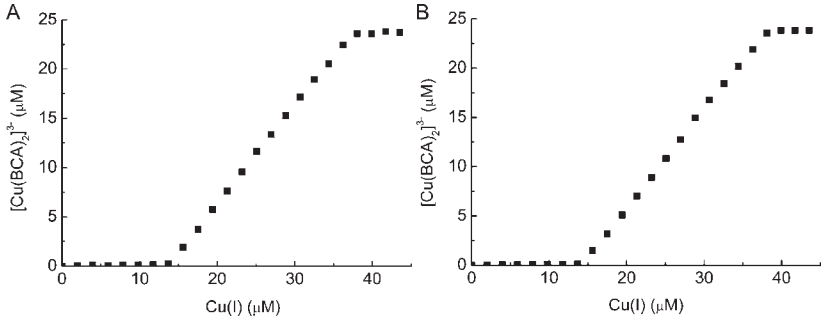

Figure 6. Plots showing the formation of $[Cu(BCA)_2]^{3-}$ upon adding Cu(I) to a mixture of apo-mb-Met (A) or apo-FL-mb (B) with 50 μM BCA in 20 mM Hepes at pH 7.5 at 25 °C.

Table 3. Molar Absorption Coefficients (ε values) at 340 and 394 nm for apo-mbs and at 290 nm for Cu(I)-mbs[a]

	ε ($mM^{-1}cm^{-1}$)		
	apo		Cu(I)
mb	340 nm	394 nm	290 nm
mb-Met	22.9 (±0.1)	22.1 (±1.5)	19.5 (±0.2)
FL-mb	21.8 (±0.1)	19.5 (±0.8)	16.4 (±0.2)

[a] The values for the apo-mbs are the average of 5 determinations, while those for Cu(I)-mbs are an average from 10 different preparations (the observed variations are shown in parentheses).

peptides, the copper is coordinated by the nitrogens from two oxazolone rings and the sulfurs from the two enethiolate groups, in a distorted tetrahedral arrangement (Figure 4 and Figure S4 and Table S1 in the Supporting Information), which is not influenced by the loss of the Met. The Cu(I) site is well protected from the solvent in both mb-Met and FL-mb. A number of interactions help to stabilize the Cu(I) site, including a hydrogen bond between the backbone amide of Cys3 and the coordinating S^{13} atom and a π interaction[38] between the first oxazolone ring (oxazolone A) and the phenol moiety of the Tyr residue. The other coordinating sulfur atom (S^{24}) is involved in a hydrogen-bonding contact with the backbone amide of Met7 in Cu(I)-FL-mb, an interaction that is absent in Cu(I)-mb-Met, where a water molecule hydrogen bonds to S^{24}. The coordinating nitrogen and sulfur ligands are linked by two carbon atoms, and thus two five-membered chelate rings form when mb binds to the metal.

Spectroscopic Properties and Metal Uptake. The UV−vis spectra of the apo- and the as-isolated Cu(I)-mbs shown in Figure 5 are similar to those reported previously.[19,20,23] For the studies described herein, accurate concentrations were essential, and we have reinvestigated the molar extinction coefficients of FL-mb as well as determined values for mb-Met. Concentrations were obtained by titrating Cu(I) into a mixture of apo-mb and a known concentration of the chromophoric Cu(I) ligand BCA. The addition of Cu(I) results in no increase in absorbance at 562 nm due to $[Cu(BCA)_2]^{3-}$ until a certain concentration when a linear increase is observed (Figure 6). Given that the Cu(I) affinity of both forms of mb is significantly greater than that of BCA (*vide infra*), the concentration of Cu(I) added before the onset of $[Cu(BCA)_2]^{3-}$ formation is equal to the concentration of apo-mb. These values were used to obtain the molar extinction coefficients shown in Figure 5A and Table 3 and were verified by Cu(II) uptake experiments analyzed by analytical HPLC (Figure 7) and EPR (*vide infra*), and also from thiol quantification of the reduced apo-peptides (*vide infra*). Copper concentrations of FL-mb and mb-Met samples isolated with bound Cu(I) were determined by AAS, giving the molar extinction coefficients shown in Figure 5B and Table 3.

X-band EPR spectra of the two *M. trichosporium* OB3b mbs isolated with copper bound show a weak, poorly resolved signal, due to a small amount of Cu(II) (Figure 8A). This signal is lost when the samples are incubated with 10 equivalents of EDTA and repurified by HPLC. Treatment with 10% nitric acid generates a strong Cu(II)

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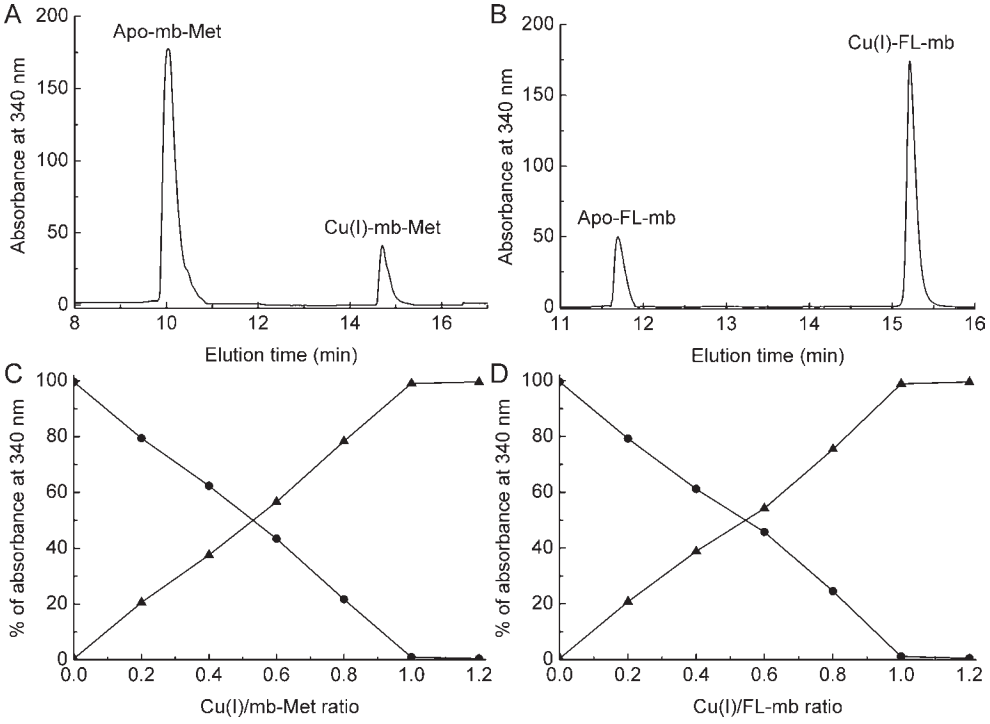

Figure 7. Copper uptake monitored by analytical HPLC. Example chromatograms obtained after injecting apo-mb-Met (30 μM) incubated with 0.2 equivalents of Cu(II) (A) and apo-FL-mb (30 μM) incubated with 0.8 equivalents of Cu(II) (B) are shown. The percentage of apo-mb and Cu(I)-mb, obtained from the intensity of the HPLC peaks, as a function of the added Cu(II)/mb ratio are shown for mb-Met (C) and FL-mb (D) with circles and triangles representing apo and Cu(I) forms, respectively.

signal (Figure 8B and C) confirming that the majority of the copper is bound as Cu(I) for both FL-mb[21,22] and mb-Met. The reduction of Cu(II)-mb-Met monitored by EPR spectroscopy (Figure 9) and UV−vis (Figure 10) is relatively fast under our experimental conditions and is complete in 10 min (analogous to what has been found previously for FL-mb by EPR[22]). The EPR spectrum alters slightly, indicating that the Cu(II) site structure is modified during this process. Simulation of the EPR spectrum that immediately forms upon Cu(II) addition to apo-mb-Met gives $g_{\|}$ and $A_{\|}$ values of 2.298 and 165 G ($g_{\perp}$ = 2.070), with $g_{\|}$ higher than what is typically expected for Cu(II) in an N_2S_2 environment.[39] However, caution is required when using EPR parameters alone to identify coordination environments, as the signal observed upon the addition of greater than ~1 equivalent of Cu(II) to apo-mb-Met (*vide infra*), in samples that were left for >10 min prior to freezing (Figure S5 in the Supporting Information), has $g_{\|}$ and $A_{\|}$ values of 2.231 and 170 G, more consistent with N_2S_2 coordination,[39] which cannot be the case. The UV−vis spectral changes upon Cu(II) addition vary with wavelength (Figure 10A and B). A rapid, large decrease in the intensity of the band at 394 nm, which is thought to derive from oxazolone A,[19] is accompanied by a smaller decrease and shift in the 340 nm band (oxazolone B[19]). A slower further decrease in the intensity of the 340 nm band occurs, which is concurrent with the disappearance of the EPR signal. The addition of Cu(I) results in relatively rapid changes at both wavelengths with the final spectrum indistinguishable from that recorded 10 min after the addition of Cu(II) (Figure 10). To determine if the addition of Cu(II) and Cu(I) to the apo-mbs resulted in the same product, ESI-MS of samples loaded with the two oxidation states of the metal were compared. The masses of both are identical to that of the molecule isolated with copper bound [it has previously been shown that the masses of FL-mb isolated from spent media to which Cu(II) and Cu(I) were added are identical[21]], and the data for FL-mb are compared in Figure S3B, C, and D in the Supporting Information.

The influence of increasing the Cu(II) and Cu(I) concentration on the UV−vis spectrum of apo-mb-Met is shown in Figure 11 and Figure S6A and C in the Supporting Information. The results are almost identical for both oxidation states of the metal [Cu(II) samples were allowed to equilibrate for ~10 min prior to measurements, allowing reduction to be complete] and also for the same experiments with FL-mb (Figure S6B and D and Figure S7 in the Supporting Information). In all cases, the major changes occur up to ~1 equivalent of copper, particularly for the peak at 340 nm. The decrease in the intensity of the peak at 394 nm continues after the addition of 1 equivalent of Cu(II), but the absorbance changes are much smaller. The addition of 1 equivalent of EDTA to a sample which had been exposed to 2 equivalents of Cu(II) returns the absorbance back to the value observed at 1 equivalent of copper. There appears to be a second site for Cu(II) that preferentially influences the 394 nm chromophore (oxazolone A), and which is also responsible for the EPR signal observed when >0.9 equivalents of Cu(II) have been added to the apo-peptides (Figure S5A and B in the Supporting Information) and possibly corresponds to

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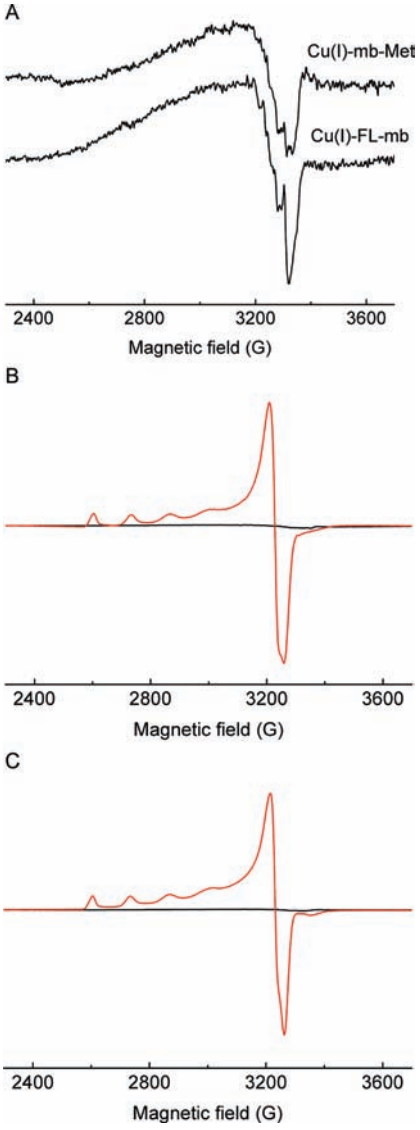

Figure 8. (A) EPR spectra (20 K) of as-isolated copper-containing mbs (2 mM) indicating the presence of a small amount of Cu(II). These samples were subsequently treated with 10% HNO_3, giving the spectra shown in red for mb-Met (B) and FL-mb (C). The spectra of the as-isolated samples (black lines) are included in B and C for comparison at an intensity 100 times less than in A.

the paramagnetic form seen in the mbs isolated with copper (Figure 8A). This second equivalent of Cu(II) is not reduced in the presence of FL-mb or mb-Met (Figure S5). Subtle differences are found for the spectra of the two forms of mb with 1 equivalent of copper added (Figure 10), which are analogous to those seen for the peptides isolated with Cu(I) bound (Figure 5B). The binding of Zn(II) to mb-Met and FL-mb (Figure S8) results in a significant decrease in the peak at 394 nm and a small increase at 340 nm [these could be indicative of the spectral changes expected upon Cu(II) binding].

The influence of pH on the UV−vis spectra of apo- and Cu(I)-mbs has also been studied. In the apo forms, the 340 nm peak (oxazolone B) is more influenced by lowering the pH, as found in methanolysis experiments,[19] and this effect is enhanced in mb-Met (denaturation starts as soon as the pH is lowered, and ~30 and 75% decreases in

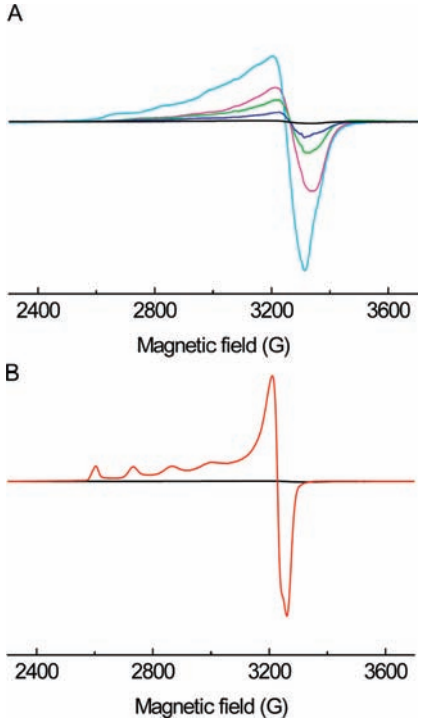

Figure 9. (A) EPR spectra (20 K) of mb-Met (1 mM) following the addition of 0.8 equivalents of Cu(II) recorded after incubating at room temperature for 10 s (cyan line), 1 min (magenta line), 2 min (green line), 5 min (blue line), and 10 min (black line). (B) An overlay of the EPR spectra of mb-Met incubated for 10 min with 0.8 equivalents of Cu(II) before (black line) and after (red line) treatment with 10% HNO_3. The intensity of the spectra in A have been multiplied by 3.1 compared to those in B.

intensity at pH 5.5 and 4.5, respectively, from pH 7.5 are seen in samples left for 20 h compared to 15 and 50% decreases, respectively, for FL-mb). Both Cu(I)-mbs are more resistant to acidic pH.

The Oxidation State of the Cysteine Residues in mbs. Both apo and Cu(I) forms (samples isolated with copper bound and also those prepared from apo-peptide) of FL-mb and mb-Met did not react with DTNB, indicating that in all cases, the two Cys residues are involved in a disulfide bond. Apo-mbs reduced with TCEP react rapidly with DTNB (reaction is complete in <1 min), with the end point indicating that 97.5−99.5% of the thiols have reacted (based on the molar extinction coefficients in Table 3). The UV−visible spectra of the reduced apo-mbs (Figure S9 in the Supporting Information) are very similar to those of the as-isolated apo forms (Figure 5A), as are the titrations of the reduced peptides with up to 1 equivalent of Cu(I) (Figure 12). The reduced mbs can bind a second, lower affinity (*vide infra*) Cu(I) ion via the two Cys residues (Figure S10 in the Supporting Information).

Cu(I) Affinities of mbs. The incubation of both as-isolated copper-mbs with 1000 equivalents of EDTA for 24 h resulted in almost no loss of metal, consistent with the presence of Cu(I). As Cu(II) is rapidly reduced in the presence of both mbs, Cu(II) affinities cannot be precisely determined, and a meaningful affinity can only be measured for Cu(I). We have determined the Cu(I) affinity of FL-mb and mb-Met by competition experiments with BCS. These experiments were performed by titrating apo-mbs into $[Cu(BCS)_2]^{3-}$ (Figure 13A) and also BCS into as-isolated Cu(I)-mbs (Figure 13B). In the latter

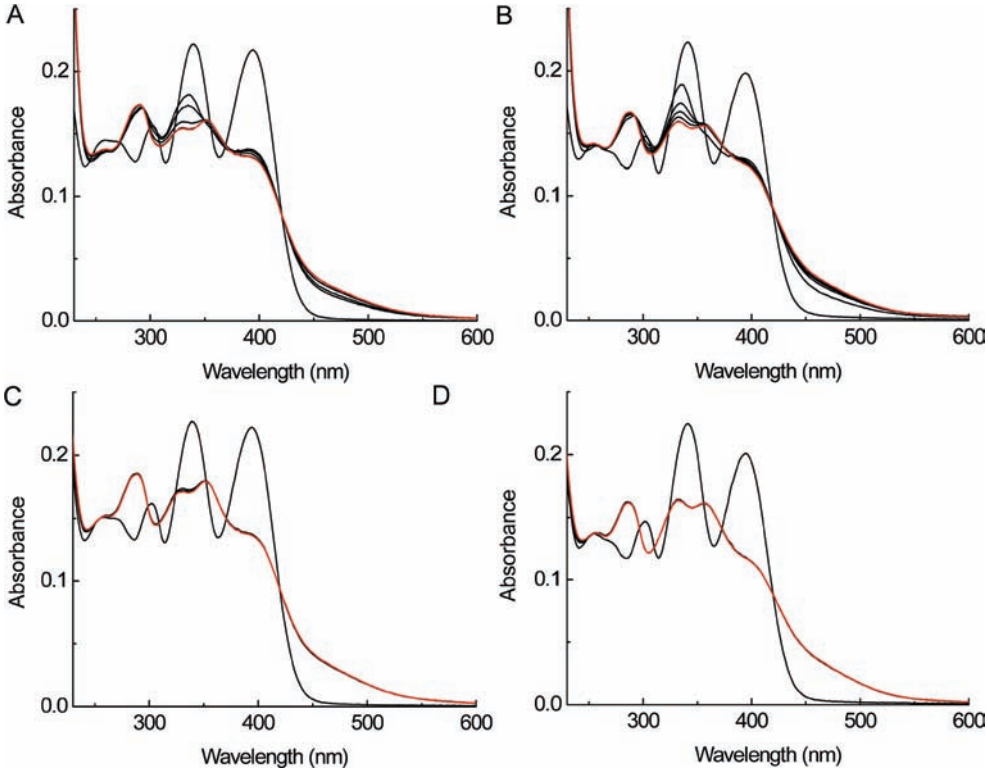

Figure 10. UV−vis spectral changes (25 °C) upon the addition of Cu(II) (A and B, respectively) and Cu(I) (C and D, respectively) to apo-mb-Met and apo-FL-mb. In all cases, the peptide (∼ 10 μM) was in 20 mM Hepes at pH 7.5, and ∼1 equivalent of copper was added. Spectra were recorded approximately 20 s, 40 s, 1 min, 2 min, 5 min, and 10 min (red line) after the addition of copper.

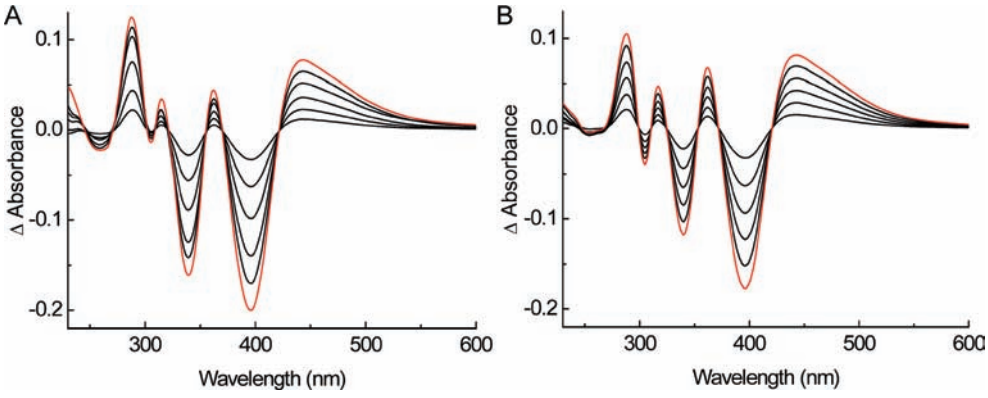

Figure 11. UV−vis difference spectra (25 °C) showing the influence of titrating mb-Met with 0 to 1 equivalents of Cu(II) (A; 25 μM mb) and Cu(I) (B; 21 μM mb) in 20 mM Hepes at pH 7.5. The red line represents the end point in each titration.

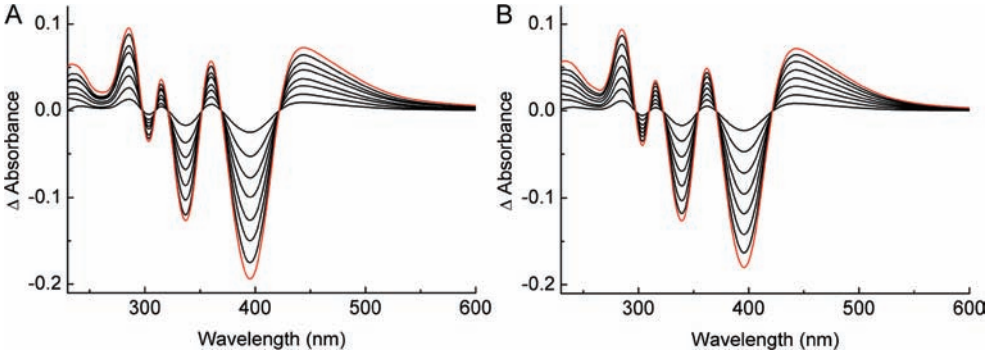

Figure 12. UV−vis difference spectra (25 °C) showing the influence of titrating 0 to 1 equivalents of Cu(I) into apo-mb-Met (A) and apo-FL-mb (B) in which the disulfide bond has been reduced (both 21 μM) in 20 mM Hepes at pH 7.5. The red line represents the end point in each titration.

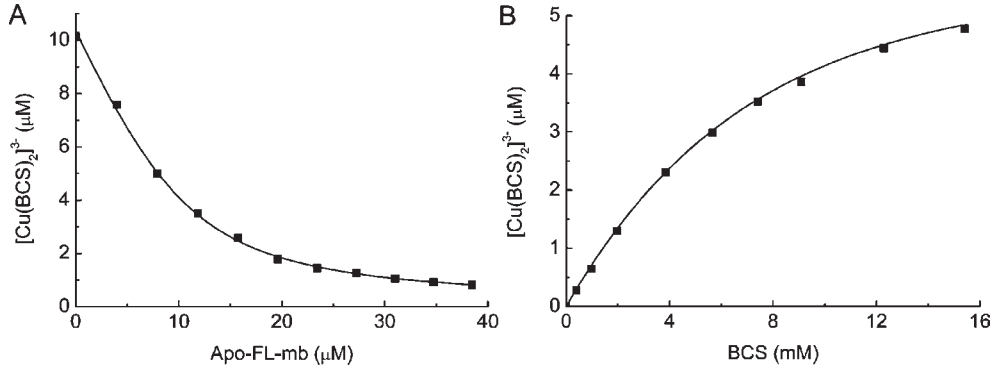

Figure 13. Titrations of (A) apo-FL-mb into a mixture of BCS (5 mM) and Cu(I) (10.2 μM) and (B) BCS into Cu(I)-mb-Met (5.8 μM) in 20 mM Hepes at pH 7.5 at 25 °C. The black lines are fits of the data to eq 2, giving K_b values of $(6.6 \pm 0.2) \times 10^{20}$ and $(6.3 \pm 0.1) \times 10^{20}$ M^{-1}, respectively.

Table 4. Cu(I) Affinities (K_b values) of mbs Determined from Competition Experiments with BCS

		K_b (M^{-1})[a]		
mb	pH	mb into $[Cu(BCS)_2]^{3-}$	BCS into Cu(I)-mb	BCS into apo-mb loaded with 0.6−0.7 Cu(II)
mb-Met[b]	6.0		$(7.5 \pm 0.4) \times 10^{19}$	
mb-Met[b]	6.5		$(1.4 \pm 0.3) \times 10^{20}$	
mb-Met[b]	7.0		$(3.6 \pm 0.4) \times 10^{20}$	
mb-Met[b]	7.5		$(5.7 \pm 0.3) \times 10^{20}$	
mb-Met[c]	7.5	$(6.7 \pm 0.3) \times 10^{20}$	$(6.1 \pm 0.2) \times 10^{20}$	$(6.6 \pm 0.2) \times 10^{20}$
mb-Met reduced[c,d]	7.5	$(8.9 \pm 0.4) \times 10^{18}$		
FL-mb[c]	7.5	$(6.8 \pm 0.2) \times 10^{20}$	$(6.2 \pm 0.3) \times 10^{20}$	$(6.5 \pm 0.3) \times 10^{20}$
FL-mb reduced[c,d]	7.5	$(8.8 \pm 0.3) \times 10^{18}$		
mb-Met[b]	8.5		$(6.3 \pm 0.3) \times 10^{20}$	
mb-Met[b]	9.5		$(6.5 \pm 0.3) \times 10^{20}$	
mb-Met[b]	10.5		$(6.4 \pm 0.3) \times 10^{20}$	

[a] All values are the average of at least three independent measurements at 25 °C with errors indicated. [b] Experiment performed in buffer (see Experimental Section) plus 200 mM NaCl. [c] Experiments performed in 20 mM Hepes at pH 7.5. [d] Samples in which the disulfide bond has been reduced.

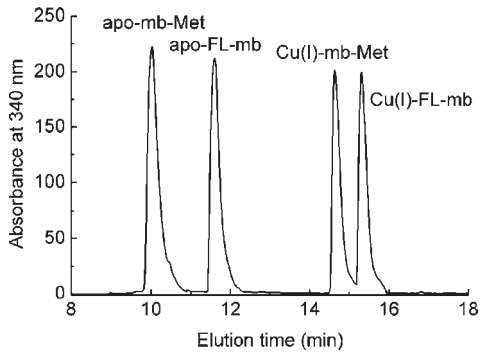

Figure 14. Analytical HPLC separation of a mixture of apo-mb-Met (50 μM) and apo-FL-mb (50 μM) incubated with Cu(II) (50 μM) in 20 mM Hepes at pH 7.5.

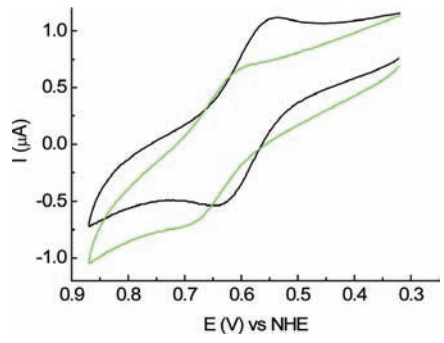

Figure 15. Cyclic voltammograms (25 ± 1 °C) of copper-loaded FL-mb (green) and mb-Met (black) in 20 mM Tris at pH 7.0 plus 90 mM NaCl at a scan rate of 20 mV/s.

experiment, > 12 mM BCS is needed to remove 75% of the copper from 5.8 μM Cu(I)-mb-Met. The Cu(I) affinity is therefore very high, and K_b values of $(6-7) \times 10^{20}$ M^{-1} are obtained at pH 7.5 for the mbs in titrations performed in both directions (Table 4). Almost identical affinities are found in experiments in which the Cu(I)-mb was prepared by the addition of 0.6−0.7 equivalents of Cu(II) to the isolated apo forms (Table 4). The similarity of the Cu(I) affinities of the mbs have been verified by equilibrating an equimolar mixture of apo-FL-mb, apo-mb-Met, and Cu(II). Separation of the mixture by analytical HPLC (Figure 14) shows an equal distribution of copper between the two forms of the molecule. In experiments where one apo-peptide was mixed with the Cu(I) form of the other mb, copper was exchanged. The final distribution was as expected considering equal affinities, but the reaction takes hours to reach equilibrium.

The influence of pH on the Cu(I) affinity of mb-Met has been investigated. These studies were performed with 200 mM NaCl present, which results in faster equilibration of Cu(I) between apo-mb and BCS but has almost no effect on the affinity for Cu(I) (Table 4). These data demonstrate that below pH 8 the affinity starts to decrease, and by pH 6 (the apo-peptides are unstable at lower pH), K_b is approximately 1 order of magnitude smaller than the maximum value (Table 4). The fit of these data to eq 4 (Figure S11 in the Supporting Information) gives a pK_a of 7.0 for the

Table 5. Ratio of *mmoX*/*pmoA* Transcript Levels in *M. trichosporium* OB3b 10 Minutes after Addition of the mbs and $CuCl_2$[a]

	log(*mmoX*/*pmoA* ratio)
	1.90 (±0.21)
$CuCl_2$	0.75 (±0.15)
apo-mb-Met	1.72 (±0.10)
Cu(I)-mb-Met	1.08 (±0.10)
apo-FL-mb	1.86 (±0.15)
Cu(I)-FL-mb	0.77 (±0.06)

[a] In all cases, additions were made to a final concentration of 2 μM. The 95% confidence intervals are denoted in parentheses. Additions of Cu(I)-mb-Met, Cu(I)-FL-mb, and $CuCl_2$ all significantly reduced the expression ratio (ANOVA; $F_{3,12} = 24.0$; $p < 0.001$), and the results are not significantly different.

single protonation event that influences the Cu(I) affinity in this range, and a K_b^{max} of $(6.6 \pm 0.4) \times 10^{20}$. The Cu(I) affinities of FL-mb and mb-Met in which the disulfide has been reduced are almost identical and are approximately 2 orders of magnitude smaller than for the forms possessing the disulfide (Table 4). The second Cu(I) site in the reduced mbs (Figure S10 in the Supporting Information) has a K_b of $\sim 3 \times 10^{15}$ M^{-1} (Figure S12 and Table S2 in the Supporting Information).

Reduction Potentials. Copper-loaded FL-mb and mb-Met give quasi-reversible electrochemical responses on a modified gold electrode (the response is better for mb-Met), and representative cyclic voltammograms are shown in Figure 15 (no signal was observed for apo-mb). Cathodic and anodic peak currents are approximately equal and show a linear dependence on (scan rate)$^{1/2}$ in the 2−100 mV/s range (Figure S13 in the Supporting Information), with peak separations of 60−80 mV at a scan rate of 20 mV/s. The average of the anodic and cathodic peak potentials can be assumed to be E_m, and values of 640 mV and 590 mV are obtained for copper-loaded FL-mb and mb-Met, respectively, at pH 7.5.

Copper-Uptake Mediated by *M. trichosporium* OB3b mbs. In previous work investigating the influence of Cu(I)-mb on the expression of pMMO versus sMMO in *M. trichosporium* OB3b cells, it was unclear whether the effects of Cu(I)-FL-mb or Cu(I)-mb-Met, or a mixture of the two, were being observed.[14] Furthermore, if previous data were obtained for Cu(I)-FL-mb, we wanted to know if Cu(I)-mb-Met has a similar effect on copper−cell interactions. Therefore, relative *pmoA* and *mmoX* transcription was quantified in the presence of both apo- and Cu(I)-mbs and also using $CuCl_2$ as the source of copper, to assess relative patterns of MMO gene expression. Copper provided as $CuCl_2$, or bound to the mbs, rapidly increases *pmoA* and reduces *mmoX* transcript levels (reported as the *mmoX*/*pmoA* ratio; post-ANOVA analysis; $p < 0.005$), showing mediated switchover from *mmoX* to *pmoA* expression, consistent with previous results.[14] No significant difference is seen in the resulting *mmoX*/*pmoA* transcript ratios ($p = 0.215$; Table 5) for Cu(I)-FL-mb, Cu(I)-mb-Met, and Cu(II) alone. This implies that both molecules are equally effective at mediating copper supply to the MMO regulatory system in *M. trichosporium* OB3b. The data with Cu(II) alone suggest that mb is not needed to promote MMO switchover at low concentrations of the metal. However, the cells will produce mb in the presence of 2 μM $CuCl_2$, which could sequester the metal and facilitate uptake [the exchange of copper between mbs is very slow (*vide supra*), and so this secreted mb should not interfere in the experiments in which Cu(I)-mbs have been used]. Mbs may become more important when copper concentrations are higher or when the metal is comparatively less available.[14,20]

Discussion

We have fully characterized two copper binding peptides (mbs) produced by *M. trichosporium* OB3b which differ by the presence or absence of a Met residue at the C-terminus. The relative amounts of the two mbs in a given culture vary, and we have shown that mb-Met is not produced by the cleavage of FL-mb by possible proteases in the media. The removal of the Met does not influence the Cu(I) site structure or overall fold, and both mbs have almost identical affinities for Cu(I). The reduction potential of the copper-loaded mbs do differ by 50 mV, which indicates different Cu(II) affinities and modified functionality (*vide infra*). The two forms allow copper uptake and the switch from sMMO to pMMO in *M. trichosporium* OB3b to be observed. We suggest that the presence of two functional mbs in *M. trichosporium* OB3b is analogous to what has been seen for organisms producing peptidic siderophores, mainly pyoverdines, where modified forms (requiring enzyme catalyzed transformations) with similar functionality are produced by a single species (the reason for producing pyoverdine isoforms is not known).[24] This comparison to pyoverdines is particularly noteworthy because mb was originally named on the basis of its structural similarities to members of this family of siderophores.[2] In fact, the mechanism of synthesis of mbs may be similar to those of peptidic siderophores,[3] which involve nonribosomal peptide synthetase (NRPS) systems.[40,41] Genetic evidence suggests NRPS systems exist in *M. capsulatus* (Bath),[42] which also produces a mb.[15,17] It should be noted that it is not uncommon for NRPS products to be modified.[43] Alternatively, mbs may arise from the modification of a ribosomally synthesized peptide.[18]

Our detailed analysis of copper uptake by FL-mb and mb-Met demonstrates that 1:1 Cu(I)-mb complexes form in both cases with the metal in a N_2S_2 (oxazolone and enethiolate groups) distorted tetrahedral site. In our experiments, UV−vis spectral changes stop after the addition of approximately 1 equivalent of Cu(II), which disagrees with what has been found previously (spectral changes stopped after the addition of ~0.5 equivalents).[23] For Cu(I) additions, our data agree with previous studies[23] showing that UV−vis spectral changes occur up to 1 equivalent. However, in our

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experiments, Cu(I) binding to apo-mb-Met and apo-FL-mb is much faster than in the earlier studies.[23] Cu(II) binding results in decreases in intensity of the peaks at both 394 and 340 nm, with the largest effect at the peak due to oxazolone A (that at 394 nm). The binding of Zn(II), and most other divalent metal ions,[44] also has a larger effect on this peak. Mbs may be used for binding other metals, although the extremely high affinity for Cu(I) indicates that copper is linked to the key function(s) of this molecule. Cu(II) is reduced in the presence of mbs, and this process occurs on a similar time scale for mb-Met and FL-mb and seems to primarily influence oxazolone B. Cu(II) binding (and reduction) has been suggested to proceed via multiple steps, and mechanisms involving dimeric and tetrameric forms have been proposed.[23,44] We show that the Cys residues are not involved in Cu(II) reduction, as a disulfide bridge is present in the apo and Cu(I) forms of both mbs, and also in Cu(II)-mbs in which the metal has been allowed to reduce. We demonstrate that the same mass is found for as-isolated Cu(I)-mbs and for purified apo-mbs loaded with Cu(II) and Cu(I). The high E_m values of the copper-mbs are very similar to that of $[Cu(BCS)_2]^{3-}$ (620 mV),[35] which can oxidize a range of amine buffers.[45] High reduction potentials are found for certain type 1 (T1) copper sites, which are known to function in biological electron transfer.[46] The multicopper oxidase ceruloplasmin has a T1 copper center which cannot be oxidized, and a lower limit of ~1 V has been reported for the E_m value of this site.[47] Cu(II)-mb from *Methylocystis* SB2 also undergoes copper reduction,[18] and this reactivity seems to be a common feature of this family of molecules under the conditions used in these experiments (El Ghazouani and Dennison, unpublished data), although the reductant has yet to be identified.

The N_2S_2 sites of FL-mb and mb-Met have affinities for Cu(I) of $(6-7) \times 10^{20}$ M^{-1}, approximately 2−3 orders of magnitude tighter than the Cu(I) site involving two Cys ligands in the archetypal cytosolic metallochaperone Atx1.[34,35] The two Cys residues in the disulfide-reduced mbs bind Cu(I) with an affinity approximately 2 orders of magnitude weaker than that of Atx1 (i.e., ~5 orders of magnitude lower than for the N_2S_2 site). The Cu(I) affinity of the N_2S_2 site in the mbs is also greater than that for the small molecule copper ligand thought to be involved in copper homeostasis in eukaryotes,[48] which has a Cu(I) affinity of $\sim 10^{16}$ M^{-1}. The E_m values for copper-loaded FL-mb and mb-Met, 640 and 590 mV, respectively, are in the range of those for related model compounds[49] as well as $[Cu(BCS)_2]^{3-}$.[35] This confirms that the tetrahedral N_2S_2 copper site of mb greatly favors Cu(I) over Cu(II) (quantified below). This stabilization of Cu(I) will prevent uncontrolled redox reactions that can give rise to toxic species, one of the suggested physiological functions of mbs[14,20] and also copper metallochaperones.[50−54]

The removal of the Met lowers the E_m of mb by 50 mV. This difference can be attributed to the loss of the hydrogen bond to S^{24} from the backbone amide of Met7, which would stabilize the Cu(II) form.[55] Small differences in the solvent accessibility of the site could also have an influence on the E_m value. It has been suggested that *M. trichosporium* OB3b mb enhances the activity of pMMO from *M. capsulatus* (Bath) by increasing electron flow to pMMO.[22] Experiments have not been reported for the reaction of a mb and pMMO from the same organism,[3] and for electron transfer to be favorable the electron accepting site in pMMO would need to have a similarly high E_m value. The influence of the removal of the Met on the E_m value means that the electron transfer reactivity of the two forms will differ. The E_m values, along with the affinities for Cu(I), allow Cu(II) affinities of 3.4×10^{12} M^{-1} and 2.1×10^{13} M^{-1} to be calculated[56] for the N_2S_2 site in FL-mb and mb-Met, respectively, at pH 7.5, which are weaker than those previously measured [as mentioned earlier, the reduction of Cu(II) in the presence of mbs precludes the determination of a meaningful Cu(II) affinity].[13,23,44] The shortened form of the molecule has a higher affinity for Cu(II), and the production of mb-Met may therefore be related to enhancing copper sequestration under certain conditions. A second weaker site for Cu(II) is present in both mbs that could involve the ring oxygen of oxazolone A and the adjacent carbonyl oxygen.

The N_2S_2 site of mb not only has a high affinity for Cu(I) but is coordinatively saturated and relatively inaccessible, which will hinder metal release via ligand exchange, the mechanism of transfer for copper metallochaperones.[50−52] In siderophores, reduction of Fe(III) is the most common mechanism of metal removal,[1] but this cannot be the case for Cu(I)-mbs. The reducing environment within a cell will promote cleavage of the disulfide bond that decreases the Cu(I) affinity of the *M. trichosporium* OB3b mbs, an effect which is probably largely entropic in origin, making Cu(I) release more favorable. The Cu(I) affinity of the N_2S_2 site of mb with the disulfide reduced is still very high, and unassisted Cu(I) removal will not occur. However, this form of the peptide should be more flexible and may have a more accessible Cu(I) site. Dissociation of one of the modified residues would result in the copper site being much more available for transfer.

Conclusions

We show that the *M. trichosporium* OB3b mbs bind Cu(I) and Cu(II) with high affinity, consistent with a role for these molecules in scavenging copper from the environment, and minimizing its toxicity. High E_m values are obtained,

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consistent with the distorted tetrahedral N_2S_2 site favoring Cu(I) over Cu(II). The lower E_m value of copper-loaded mb-Met indicates a possible functional difference in terms of redox activity or Cu(II) affinity. Both mbs mediate copper-regulated switchover from sMMO to pMMO in *M. trichosporium* OB3b. Given the extremely high affinity for Cu(I), specific release mechanisms for copper from mb will be necessary.

Acknowledgment. We thank NERC (grant NE/F0060-8X/1) for funding and Dr. Adriana Badarau for helpful discussions particularly about copper affinity determinations, Dr. Ernest Chi-Fru for providing some crude mb extracts, and Dr. Neil Gray for useful discussions.

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Supporting Information Available: Figures showing chemical structures, analytical HPLC chromatograms, MS data, electron density maps, copper binding experiments analyzed by EPR and UV−vis, Zn(II) binding, the spectral properties of mbs with the disulfide reduced, the pH dependence of K_b for mb-Met, an affinity determination for the weaker Cu(I) site in reduced mb-Met, the scan rate dependence of peaks current for mb-Met, and tables comparing the Cu(I) centers in the mbs and the affinity of the weak site. This material is available free of charge via the Internet at http://pubs.acs.org.