Spectral and Thermodynamic Properties of Au(III), Cd(II), Co(II), Fe(II), Fe(III),
Hg(II), Mn(II), Ni(II), U(IV), and Zn(II) Binding by Methanobactin from

*Methylosinus trichosporium* OB3b†

Dong W. Choi¹, Young S. Do¹, Corbin J. Zea², Sung-W. Lee³, Nicola L. Pohl², Jeremy D. Semrau³,

Clint J. Kisting¹, Marcus T. McEllistrem⁴, Eric S. Boyd⁵, Gill G. Gessey⁵,

William E. Antholine⁶, and Alan A. DiSpirito¹*

¹Department of Biochemistry, Biophysics and Molecular Biology and ²Department of Chemistry and the

Plant Sciences Institute, Iowa State University, Ames, IA, 50011-3211

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

2125

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

⁵Department of Microbiology, Montana State University, Bozeman, MT 59717

⁶Department of Biophysics, Medical College of Wisconsin, Milwaukee, WI 53226

AUTHOR EMAIL ADDRESSES Dong W. Choi (dwon@iastate.edu), Corbin J. Zea (czea@gvc.edu), Young S. Do (youngsdo@yahoo.com), Sung-W. Lee (jsemrau@engin.umich.edu), Jeremy D. Semrau (jsemrau@engin.umich.edu), William E. Antholine (wantholi@mcw.edu), Clint J. Kisting (celeeto78@yahoo.com), Marcus T. McEllistrem (mcellimt@uwec.edu), Eric S. Boyd (eboyd@montana.edu), Gill G. Gessey (gill_g@erc.montana.edu), and Alan A. DiSpirito (aland@iastate.edu)

†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

TITLE RUNNING HEAD: Metal-binding by methanobactin

CORRESPONDING AUTHOR FOOTNOTE: A.A. DiSpirito, Tel: 515-294-2944, Fax: 515-294-0453

E-mail address: aland@iastate.edu
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, fluorescent, 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 Au(III), Co(II), Cd(II), Fe(III), Hg(II), Mn(II), Ni(II), U(VI), or Zn(II) but not Ca(II), Cr(IV) and Mg(II). However, the binding constants were less than those observed with Cu(II) and copper displaced other metals bound to mb, suggesting mb is specifically involved in copper metabolism. With the possible exceptions of Au(III) and Hg(II), the coordination of these metals differs from that observed with Cu(I) or Cu(II). Again with the exception of Au(III) and possibly Hg(II), and in contrast to Cu(II), none of the metals examined were reduced by mb. Thus, although mb will bind a variety of metals, the results are consistent with it’s role as a copper-binding compound or chalkophore.
KEYWORDS: chalkophore; copper-binding compound; methanobactin; membrane-associated methane monooxygenase; methanotroph.

Abbreviations: ΔA, absorption change; CT, charge transfer; Cu-mb, copper-containing methanobactin; EPR, electron paramagnetic resonance; ITC, isothermal titration calorimetry; K, binding constant; mb, methanobactin; mb₂, homodimer of methanobactin; MMO, methane monooxygenase; pMMO, membrane-associated methane monooxygenase; sMMO, soluble methane monooxygenase; XPS, X-ray photoelectron spectroscopy.
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 (Figure 1, reaction 1). The UV-visible absorption spectra associated with this initial step showed a decrease in the absorption maxima associated THI, at 394 nm (Figures 1 and 2). This initial coordination was followed by a reduction of Cu(II) to Cu(I) (Figure 1, reaction 2), which was then followed by a change in metal ligation resulting in coordination by both THI and the 4-hydroxy-5-thiocarbonyl imidazolate (HTI) (Figure 1, reaction 3). Coordination of Cu(II) or Cu(I) to HTI resulted in a decrease in intensity at 340 nm (Figure 2). At Cu(II) to mb ratios above 0.25 the tetramer of oligomer dissociates into a dimer (Figure 1, reaction 4). Lastly, at
copper concentrations above 0.5 Cu per mb, the dimer dissociates into monomers (Figure 1, reactions 5 and 6) resulting in one Cu(I) atom bound per mb. At copper to mb ratios above 1 Cu(II) per mb, a new round of binding was observed with the release of insoluble Cu(I).

The structural similarities of mb to siderophores in the pyroverdin 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 spectral and thermodynamic properties of Au(III), Co(II), Cd(II), Fe(III), Fe(II), Hg(II), Mn(II), Ni(II), and U(VI) binding were examined. The results suggest 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.
MATERIALS AND METHODS

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). For preparations of metal saturated mb samples, CaCl₂, 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.

Metal Titrations. Metal titration experiments were determined by addition of 100 µM, 1 mM, or 10 mM solutions of HAuCl₄, CaCl₂, CdCl₂, CoCl₂, CrO₂, CuSO₄, FeCl₃, HgCl₂, MgCl₂, MnCl₂, NiCl₂, 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).

Spectroscopy, Isothermal Titration Calorimetry (ITC), and Metal Determinations. UV-visible absorption, florescent, circular dichroism (CD), electron paramagnetic resonance spectra (EPR), isothermal titration calorimetry (ITC), 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 base line was used as a reference point of 0 instead of isosbestic point e for the comparison of Δεs.

X-ray Photoelectron Spectroscopy (XPS). XPS was preformed 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). Marc and
Bill anything specific about Au, Ni and Mn XPS or Ni, Co, Zn, or Mn EPR should be added here.

*Transmission Electron Microscopy.* 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; gold to mb molar ratios of 0, 0.1, 0.2, 0.4, 0.75, 1.0, 1.5, 2.0, 3.0, and 4.0 Au(III) to mb. All samples were incubated for 15 min with continuous stirring followed by freezing at -80 °C. Au-mb solutions were spotted on parlodion coated Ni-grids, dried under vacuum and examined with a JEOL 1200X scanning/transmission electron microscope.
RESULTS

Metal Bound by Mb. Initial screening of metals bound by mb showed that in the absence of Cu(II) or Cu(I), mb will bind Au(III), Cd(II), Co(II), Fe(III), Hg(II), Mn(II), Ni(II), U(VI), or Zn(II) but not Ca(II), Cr(IV) and Mg(II). The concentration of Cd(II), Co(II), Hg(II), Mn(II), Ni(II), and Zn(II) bound to mb was approximately half of that observed with Cu(II) suggesting mb binds these metals as a dimer (Table 1). Spectral (UV-visible absorption, EPR, CD, and fluorescent) and thermodynamic studies of ferric and ferrous binding by mb suggested mb bound iron as a dimer (see below). However, the iron associated with mb dissociated from mb following H$_2$O wash used to remove excess metal on Dianion HP-20 columns (Table 1).

Due to precipitation or altered column binding properties following exposure to excess metal concentrations, the concentration of Au(III), Hg(II), and U(IV) bound by mb were determined via titration experiments (see below).

Metal Binding Groups. Based on the spectral properties described below, the metals bound by mb were placed into four groups. Choi et al. (8) recently described the spectral and thermodynamic properties of Cu(II) and Cu(I) which are designated as group A metals. Group B metals consists of Cd(II), Co(II), Fe(II), Fe(III), Ni(II), and Zn(II). Mb appears to coordinate group B metals as a dimer via THI, without a change in the metal oxidation state. With respect to the metal binding model proposed for Cu(II) (figure 1 this study; figure xxx in Choi et al.(8)), coordination of group B metals stopped after the initial binding step (figure 1). Group C metals consist of Au(III) and Hg(II). Like group A metals, the binding of group C metals by mb involves THI, HTI, and possibly Tyr. However, the final conformation of the mb following the binding of group C metals differs from that observed with group A metals. Also like group A metals, group C metals were reduced by mb. The coordination of group X metals (Mn(II) and U(IV)) have not been determined.
**UV-Visible Absorption Spectra.** The binding of group B metals by mb resulted in a decreased absorption at 394 nm, with either no change (Ni and Fe), or a small increase (Cd, Co, and Zn) in absorption at 340 nm (Figure 2 and 3A, and 3B). With the exception of Ni(II), little to no changes in absorbance were observed in the 250 – 310 nm range for this metal group. Thus, the spectral changes associated with the binding of group B metals were similar to those observed in the initial coordination to Cu(II) (8) which are consistent with a dimer binding model via the THI moieties (Figure 1, 1).

Like groups A and B metals, the binding of Au(III) and Hg(II) (group C metals) resulted in a decreased absorption at 394 nm (Figure 4A). In contrast to groups A and B metals, the spectral changes associated with HTI were complex. At low (i.e. < 0.3 metal per mb) Au(III) or Hg(II) concentrations, a red shift in the absorption maximum from 340 to 363 nm was observed with an increased absorption at 363 nm. At metal to mb ratios above 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. ≤ 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 results suggest that coordination of group C metals to HTI differs from group A metals and/or the interactions between metal and HTI varied at different metal to mb ratios.

Like group A and C metals, the addition of Mn(II) or U(IV) (group X metals) to mb resulted in spectral changes from both THI and HTI (Figure 5A and 5B). Addition of U(IV) resulted in decreased absorption at 340 and 394 with an associated increased absorption at 302 and 282 nm (results not shown). Mn(II) addition resulted in a decreased absorption at the maxima of 394 nm along with an associated blue shift to 377 nm. Mn(II) addition also resulted
in an increased absorption at the maxima of 340 nm and a decrease in the absorption at 302 nm (Figures 2, 5A and 5B). 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). With U(IV) addition, both the intensity at both 282 and 302 nm increased; again suggesting these absorption maxima do not represent the phenolic and phenoxide ion forms of Tyr.

_Fluorescence Spectroscopy._ Like group A metals (8), addition of group B and C metals quenched emissions from THI following excitation at 394 nm ($\lambda_{\text{ex}}$394) (Figures 3E, 4E, and 4F; Table 3). The addition of group X metals had no effect on emission from THI (Figures 5E and 5F). With the exception of Cd(II), the addition of groups B and C metals also quenched emission from Tyr (Figures 3E, 4E, and 4F; Table 3) suggesting Tyr was either involved in metal coordination or was proximal to the metal coordination site. As with emissions from THI, the addition of Mn(II) to mb resulted in small, increased emissions from Tyr.

The addition of groups B, C and X metals had mixed effect 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). Cation induced fluorescence has been shown to occur with removal or separation of an internal quencher following cation binding or with cation binding to the internal quencher (20, 21). Binding of group B and C metals, as well as group X metals, quenched emissions from HTI. The quenching of HTI with Co(II), Fe(III) and Ni(II) was inconsistent with the results from UV-visible absorption spectra and may result from the proximal location of these metals to HTI instead of coordination to this ligand.
Circular Dichroism Spectroscopy. The UV-CD spectrum of mb showed a strong negative band below 200 nm with negative shoulders at 202 and 217 nm (figures 3C, 4C and 5C), characteristic of an unordered polypeptide (22). Addition of group B and X metals resulted in positive band enhancements at 190 nm, suggesting the development of α-helical characteristics (figures 3 and 5). Like the UV-visible absorption spectra, the CD spectra of the group C metals were complex and depended on the metal to mb ratio (Figure 4). At metal to mb concentrations below 0.4 metal to mb, the UV-CD spectra was similar to that of group A metals (Figure 4C and 4D). However, at concentrations of metal to mb >0.5, the trend reversed.

Development of typical exciton coupled spectrum of two chromophore system (THI and HTI) were seen in wavelength range between 315 and 415 nm with all metals tested (Figures 3C, 3D, 4C, 4D, 5C, and 5D). The CD spectra in this region were consistent with a Cotton effect involving the THI and HTI (22-24). In group B and X metals, a negative band enhancement near 340 nm (2nd Cotton effect, HTI) and a positive band enhancement near 360 nm (1st Cotton effect, THI) were observed with metal addition suggesting the two chromophores were brought together with a counter-clockwise twist (positive chirality) (22-24). The absorbance maxima associated with HTI remained near 340 nm, suggesting the hydrophobicity of this group did not change. The absorption maxima associated with THI showed a blue shift indicating THI moved to a more hydrophobic environment.

The visible CD spectrum of mb following the addition of group C metals followed the complex trends observed in the UV-CD spectra (Figures 4C and 4D). At molar ratios below XXX per mb, the UV-CD spectra were similar to that observed following copper addition (8) and opposite to that observed with groups B and X metals. The CD-spectra associated with HTI following the addition of group C metals resulted in a red shift from 340 nm to 360 nm 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 these groups. At metal concentrations above 0.5 metals per mb, the trends throughout the CD-spectrum reversed. With the exception of the spectral shift of HTI to 360 nm, the spectra at 2.0 Hg(II) or Au(III) per mb were similar to metal free mb. In contrast to copper (8), no strong relationships between Tyr and HTI were observed in the CD spectra following the addition of groups B, C or X metals.

Oxidation State of Metals Bound to Mb. EPR spectra of Fe-mb and Co-mb suggested metal coordination, but not reduction (Figure 6). Similar results were obtained with mb-Ni(II), mb-Mn(II) and mb-Zn(II) (results not shown). XPS-spectroscopy of Fe-mb complexes confirmed iron associated with mb remained in the ferric state (results not shown). Thus, in contrast to Cu(II) (2, 3, 5, 8), none of the group B and X metals examined were reduced by mb. On the other hand, XPS showed that the group C metal, Au(III) metal, was reduced to Au(0) by mb. In fact, up to 6 Au(III) were reduced to Au(0). The oxidation state of Hg bound to mb was not determined, but formation of insoluble gray precipitates at Hg(II) to mb ratios above 0.8 Hg(II) per mb suggested Hg(II) was also reduced by mb.

Transmission Electron Microscopy. 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 Au(0) nanoparticles (Figure 7). However, if samples were centrifuged or subjected to one freeze-thaw cycle, Au(0) nanoparticles were observed at Au(0) to mb ratios above 0.8 Au(0) per mb. The nanoparticle sizes ranged from 5 – 30 nm with the majority (60%) in the 11 – 20 nm particle range.
Isothermal Titration Calorimetry (ITC). The binding constants observed with group B, C and X metals were below the binding constants observed with group A metals (8) (Table 4, Figure 9). Like group A metals, most of the metals examined fit a two-site binding model better than a one-site binding model. However, as a general rule, the transition in thermodynamic properties at 0.5 metal per mb was more pronounced than observed with Cu(II) (Figure 9). Most of the metals followed a titration curve similar to Hg(II) (Figure 9A) or to Ni(II) (Figure 9C) with the most extreme transitions observed with Zn(II) and Au(III). The reason for the initial increased free energy change with increased Au(III) concentration at molar ratios below 0.4 Au(III) per mb was not determined, but may be associated with the formation of Au(0) nanoparticles (Figure 8B). The reason for the a transition from exothermic to endothermic in Zn(II) titrations was not determined, but may represent dimer dissociation (Figure 7).

Oligomerization of Mb. As observed with group A metals (8), the binding of other metals by mb resulted in the formation of oligomers (Figure 9). Similar results have been shown with a variety of synthetic metal binding compounds where the addition of cations resulted in the formation of stable oligomers (25-28).
DISCUSSION

In contrast to iron siderophores, which are generally specific for Fe(III) (14, 16, 29-31), 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 uncontrollable transport of radionuclides and heavy metals from these sites to surrounding areas (32-38). Methanotrophic bacteria are often present at these sites and often used in the remediation of halogenated hydrocarbons (39). 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.

The results presented here also suggest the mechanism of binding to groups B, C and X metals differs from that observed with Cu(I) and Cu(II) (8). Mb appears to bind group B metals as a dimer via THI (Figure 1, 1). With respect to the mechanism of binding, group B metals appear to follow the initial binding step observed with group A metal (Figure 1, 1) (8). The mechanism of metal binding by group C metals showed a number of similarities to group A metals. First, at low metal concentrations, mb appeared to bind group C metals as an oligomer via both THI and HTI. Second, the binding of group C metals was followed by reduction. Third, at metal to mb ratios above 0.5 mb, mb bound Au or Hg as a monomer (figure 1, 1 – 6 or 1 – 2 and 7 – 8). Lastly, at least in the case of Au, more than one group C atom was reduced per mb. Taken together the results suggest group C metals followed a metal binding and reduction...
scheme similar group A metals (Figure 1, 1–6), although the CD-spectra suggests the final
conformation of the mb-Au or Hg-mb complex differed from that observed with group A metals.
ACKNOWLEDGEMENTS

We thank V. Frasca at Microcal for assistance in modeling of the ITC results and Tracey M. Pepper (ISU) for TEM analysis.
REFERENCES


Choi, D. W., Zea, C. J., Do, Y. S., Semrau, J. D., Antholine, W. e., Hargrove, M. S.,
Pohl, N. L., Boyd, E. S., Geesey, G. G., Hartse., S. C., Shafe, P. H., McEllistrem, M. T.,
Spectral, kinetic, and thermodynamic properties of Cu(I)-, and Cu(II)-binding by
methanobactin from *Methylosinus trichosporium* OB3b. *Biochemistry* 45, 1442 - 1453.
and Georgiou, G. (1993) Phenotypic characterization of copper-resistant mutants of
Purification and physical-chemical properties of methanobactin: a chalkophore from
Morton, J. D., Hayes, K. F., and Semrau, J. D. (2000) Bioavailability of chelated and soil-
absorbed copper to *Methylosinus trichosporium* OB3b. *Environ. Sci. Technol* 34, 4917 -
4922.
*trichosporium* OB3b mutants having constitutive expression of soluble methane
monooxygenase in the presence of high levels of copper. *Appl. Environ. Microbiol.* 58,
3701-3708.


Table 1: Molar ratios of metal per mb isolated following exposure to 100 fold molar excess of Cu(II) (Cu-mb), Fe(III) (Fe-mb), Cd(II) (Cd-mb), Zn(II) (Zn-mb), Ni(II) (Ni-mb), Mn(II) (Mn-mb), or Co(II) (Co-mb). Standard variance was equal to or less than 20%.

<table>
<thead>
<tr>
<th>Metal</th>
<th>mb</th>
<th>Cu-mb</th>
<th>Fe-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>0.0837</td>
<td>bd</td>
<td>0.0014</td>
<td>0.0010</td>
<td>0.0002</td>
<td>bd</td>
</tr>
<tr>
<td>Fe(III)</td>
<td>0.013</td>
<td>0.0100</td>
<td>0.0265</td>
<td>0.0123</td>
<td>0.0366</td>
<td>0.0234</td>
<td>0.0200</td>
<td>0.0305</td>
</tr>
<tr>
<td>Cd(II)</td>
<td>0.003</td>
<td>0.0006</td>
<td>bd</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>0.038</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>0.0113</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>0.0299</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>0.0056</td>
<td>0.0017</td>
<td>0.0004</td>
<td>0.0002</td>
<td>0.0001</td>
<td>0.8068</td>
</tr>
</tbody>
</table>

*below detection
Table 2. Molar absorption coefficients ($\varepsilon$) of mb and metal-mb.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>$\varepsilon_{340}$ (mM$^{-1}$cm$^{-1}$)</th>
<th>$\Delta\varepsilon_{340}$ (mM$^{-1}$cm$^{-1}$)</th>
<th>$\varepsilon_{394}$ (mM$^{-1}$cm$^{-1}$)</th>
<th>$\Delta\varepsilon_{394}$ (mM$^{-1}$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><strong>Group B</strong></td>
<td></td>
<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>
<td>18.60</td>
<td>-0.36*</td>
<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>
</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>
<tr>
<td><strong>Group C</strong></td>
<td></td>
<td></td>
<td></td>
<td></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><strong>Group X</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn-mb</td>
<td>20.54</td>
<td>-2.30*</td>
<td>11.28</td>
<td>4.79</td>
</tr>
<tr>
<td>U-mb</td>
<td>16.24</td>
<td>2.00</td>
<td>13.98</td>
<td>2.09</td>
</tr>
</tbody>
</table>

* An increase in absorbance was observed
Table 3: Change in emission intensities from Tyr (\(\lambda_{280}\) nm), THT (\(\lambda_{394}\) nm), and HTI (\(\lambda_{340}\) nm) following the addition of equimolar concentrations of metals to mb.

<table>
<thead>
<tr>
<th>Metal</th>
<th>Change in Emission Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tyr</td>
</tr>
<tr>
<td></td>
<td>310 nm</td>
</tr>
</tbody>
</table>

**Group A**

- **Cu(II)**
  - \(-4.88^b\)
  - \(-0.03^b\)
  - \(-3.82^b\)
  - \(-1.00^b\)
  - \(-0.83^b\)
  - \(-1.57^b\)

**Group B**

- **Cd(II)**
  - \(-0.43\)
  - \(+1.76\)
  - \(-1.72\)
- **Co(II)**
  - \(-0.10\)
  - \(-0.43\)
  - \(-1.72\)
- **Fe(III)**
  - \(-4.84\)
  - \(-0.92\)
  - \(-3.23\)
- **Ni(II)**
  - \(-2.85\)
  - \(-0.37\)
  - \(-2.70\)
- **Zn(II)**
  - \(-0.88\)
  - \(+0.52\)
  - \(-4.0\)

**Group C**

- **Hg(II)**
  - \(-4.26\)
  - \(+1.11\)
  - \(-3.82\)
- **Au(III)**
  - \(-6.36\)
  - \(+2.01\)
  - \(-3.80\)

**Group X**

- **Mn(II)**
  - \(+0.17\)
  - \(-0.44\)
  - \(+0.37\)

*from Choi *et al.* (8)

\(^b\)as isolated by Choi *et al.* (8)

\(^b\)isolated following Cu(II) saturation and NaEDTA treatment (8, 10)
Table 4. Thermodynamic parameters for metal bindings to mb at pH6.8. Subscripts 1 and 2 indicates the first binding site and second binding site, respectively.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Au(III)</th>
<th>Hg(II)</th>
<th>Fe(III)</th>
<th>Mn(II)</th>
<th>Ni(II)</th>
<th>Cd(II)</th>
<th>Co(II)</th>
<th>Zn(II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$N_1$ (metal mb$^{-1}$)</td>
<td>$0.10 \pm 0.187$</td>
<td>$0.25 \pm 0.005$</td>
<td>$0.27 \pm 0.007$</td>
<td>$0.54 \pm 0.005$</td>
<td>$0.27 \pm 0.023$</td>
<td>$0.20 \pm 0.007$</td>
<td>$0.49 \pm 0.003$</td>
<td>$0.41 \pm 0.004$</td>
</tr>
<tr>
<td>$K_1$ (M$^{-1}$)</td>
<td>$1.0 \pm 0.5 \times 10^5$</td>
<td>$9.9 \pm 2.9 \times 10^4$</td>
<td>$9.7 \pm 0.6 \times 10^4$</td>
<td>$7.7 \pm 1.8 \times 10^5$</td>
<td>$4.9 \pm 0.9 \times 10^5$</td>
<td>$1.3 \pm 0.8 \times 10^6$</td>
<td>$1.1 \pm 0.2 \times 10^6$</td>
<td>$4.5 \pm 1.4 \times 10^6$</td>
</tr>
<tr>
<td>$\Delta H_1$ (kcal mol$^{-1}$)</td>
<td>$6.7 \pm 2.6 \times 10^4$</td>
<td>$-25.1 \pm 0.2$</td>
<td>$-5.31 \pm 0.38$</td>
<td>$-0.07 \pm 0.00$</td>
<td>$-5.71 \pm 0.28$</td>
<td>$-3.15 \pm 1.69$</td>
<td>$-4.08 \pm 0.05$</td>
<td>$-0.32 \pm 0.01$</td>
</tr>
<tr>
<td>$\Delta S_1$ (cal mol$^{-1}$ deg$^{-1}$)</td>
<td>$2.2 \times 10^5$</td>
<td>$-52.3$</td>
<td>$5.0$</td>
<td>$26.7$</td>
<td>$6.9$</td>
<td>$17.4$</td>
<td>$13.9$</td>
<td>$29.4$</td>
</tr>
<tr>
<td>$\Delta G_1$ (kcal mol$^{-1}$)</td>
<td>$1.44 \times 10^3$</td>
<td>$-9.81$</td>
<td>$-6.80$</td>
<td>$-8.02$</td>
<td>$-7.75$</td>
<td>$-8.34$</td>
<td>$-8.22$</td>
<td>$-9.08$</td>
</tr>
<tr>
<td>$N_2$ (metal mb$^{-1}$)</td>
<td>$0.38 \pm 0.16$</td>
<td>$0.42 \pm 0.006$</td>
<td>$0.31 \pm 0.014$</td>
<td>-</td>
<td>$0.18 \pm 0.025$</td>
<td>$0.22 \pm 0.010$</td>
<td>-</td>
<td>$0.13 \pm 0.040$</td>
</tr>
<tr>
<td>$K_2$ (M$^{-1}$)</td>
<td>$1.8 \pm 0.2 \times 10^5$</td>
<td>$89.9 \pm 10.1$</td>
<td>$1.7 \pm 0.4 \times 10^6$</td>
<td>-</td>
<td>$1.2 \pm 0.2 \times 10^7$</td>
<td>$1.1 \pm 0.6 \times 10^7$</td>
<td>-</td>
<td>$1.8 \pm 0.0 \times 10^4$</td>
</tr>
<tr>
<td>$\Delta H_2$ (kcal mol$^{-1}$)</td>
<td>$3.10 \pm 0.69$</td>
<td>$-16.2 \pm 0.05$</td>
<td>$-8.15 \pm 0.08$</td>
<td>-</td>
<td>$-6.89 \pm 0.26$</td>
<td>$-18.96 \pm 1.04$</td>
<td>-</td>
<td>$2.40 \pm 0.78$</td>
</tr>
<tr>
<td>$\Delta S_2$ (cal mol$^{-1}$ deg$^{-1}$)</td>
<td>$34.5$</td>
<td>$-31.7$</td>
<td>$1.14$</td>
<td>-</td>
<td>$9.23$</td>
<td>$-31.3$</td>
<td>-</td>
<td>$27.5$</td>
</tr>
<tr>
<td>$\Delta G_2$ (kcal mol$^{-1}$)</td>
<td>$-7.18$</td>
<td>$-6.92$</td>
<td>$-8.49$</td>
<td>-</td>
<td>$-9.64$</td>
<td>$-9.63$</td>
<td>-</td>
<td>$-5.80$</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>$3.90 \times 10^4$</td>
<td>$1.76 \times 10^5$</td>
<td>$4.70 \times 10^3$</td>
<td>$9.39$</td>
<td>$2.82 \times 10^4$</td>
<td>$1.01 \times 10^5$</td>
<td>$1.95 \times 10^4$</td>
<td>$7.26 \times 10^6$</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1. Model for the binding of group A (Cu(II), group B (Cd(II), Co(II), Fe(III), Ni(II), and Zn(II)), and group C (Au(III) and Hg(II)), metals by mb. Mb represented as two bars ending in the N^ε atom of each imidazolate and the S atom of each thiocarbonyl group on 4-thiocarbonyl-5-hydroxy imidazolate (yellow and orange bar) and 4-hydroxy-5-thiocarbonyl imidazolate (orange bar). Abbreviations: M^o, metal in the oxidation state added and M', metal reduced by mb.

Figure 2. Difference UV-visible absorption spectra of Cu-mb minus mb (-----), Ni-mb minus mb (-----), and Mn-mb minus mb (-----).

Figure 3. (A) UV-visible absorption spectra of mb following addition of 0.1, 0.2, 0.3, 0.4, 0.6, 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). λ_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 (λ_ex = 394nm, △), 461 (λ_ex = 340nm, ●), and 310 nm (λ_ex = 280nm, ■).
Figure 4. (A) UV-visible absorption spectra of mb following addition of 0.1 to 1.0 Au(III) atoms 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 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). λ<sub>ex</sub> = 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 (λ<sub>ex</sub> = 394nm, △), 461 (λ<sub>ex</sub> = 340nm, ●), and 310 nm (λ<sub>ex</sub> = 280nm, ■).

Figure 5. (A) UV-visible absorption spectra of mb following addition of 0.1 to 2.0 Mn(II) atoms per mb. Arrows indicate the direction of spectra changes upon Mn(II) additions. (B) Absorption changes at 394 (△), 377 (●), 340 (●), 302 (■), and 254 nm (○) following Mn(II) additions. (C) CD spectra of mb as isolated (thick line) and following additions of 0.1 to 2.0 molar equivalents of Mn(II) (thin lines). (D) The effect of Mn(II) addition on the CD spectra at 364 (△), 338 (●), 302 (●), 282 (■), and 190 nm (○). (E) Emission spectra of mb in aqueous solution with different excitation wavelength (nm). λ<sub>ex</sub> = 280, 340, and 394 nm at ambient temperature (thick lines). Arrows indicate the direction of spectrum changes upon Mn(II) additions and thin lines show the spectra upon completion of changes. (F) Emission intensity changes at 610 (λ<sub>ex</sub> = 394nm, △), 461 (λ<sub>ex</sub> = 340nm, ●), and 310 nm (λ<sub>ex</sub> = 280nm, ■).
Figure 6. X-band EPR spectra at 77 K of mb (concentration 4 mM) following the addition of equimolar equivalents of Fe(III) (A) and Cu(II) (B). Experimental conditions: modulation amplitude, 5 G, modulation frequency, 100 KHz, microwave power, 5 mW, temperature 77 K.

Figure 7. Transmission electron micrographs of methanobactin solutions following the addition of 1 (A), 1.5 (B), or 2 (C) Au per mb. D, TEM of 2 Au per mb following one freeze-thaw cycle.

Figure 8. Binding isotherm of 3.2 mM HgCl$_2$ (A), HAuCl$_4$ (B), NiCl$_2$ (C), or ZnCl$_2$ (D) 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.

Figure 9. A, Separation of mb on a Superdex peptide HR 10/30 column (——), following the addition of 0.1 Hg(II) per mb (——), or 0.5 Hg(II) per mb ( - - - - ). B, Separation of mb on a Superdex peptide HR 10/30 column (——), following the addition of 0.1 Ni(II) per mb (——), or 0.5 Ni(II) per mb ( - - - - ).
1 Figure 1.

2

3 Figure 2.

4

5

6
Figure 3.
Figure 4.
Figure 5.
Figure 6

![EPR Absorption Spectra](image)

Figure 7

![TEM Images](image)
Figure 8.

Figure 9.
Spectral and Thermodynamic Properties of Au(III), Cd(II), Co(II), Fe(II), Fe(III), Hg(II), Mn(II), Ni(II), U(IV), and Zn(II) Binding by Methanobactin from

*Methylosinus trichosporium* OB3b

Dong W. Choi¹, Young S. Do¹, Corbin J. Zea², Sung-W. Lee³, Nicola L. Pohl², Jeremy D. Semrau³, Clint J. Kisting¹, Marcus T. McEllistrem⁴, and William E. Antholine⁵, Alan A. DiSpirito⁶*
Spectral and Thermodynamic Properties of Au(III), Cd(II), Co(II), Fe(II), Fe(III), Hg(II), Mn(II), Ni(II), U(IV), and Zn(II) Binding by Methanobactin from *Methylosinus trichosporium* OB3b

Dong W. Choi¹, Young S. Do¹, Corbin J. Zea², Sung-W. Lee³, Nicola L. Pohl², Jeremy D. Semrau³, Clint J. Kisting¹, Marcus T. McEllistrem⁴, and William E. Antholine⁵, Alan A. DiSpirito¹*

**Supplementary information**

Figure S1. UV-visible absorption spectra of mb following addition of 0.1 to 1.0 Cd(II) atom (A), Co (II) atom (B), Fe (III) atom (C), Hg(II) atom (D), U(IV) atom (E), or Zn(II) atom (F) per mb. Arrows indicate the direction of spectra changes and dotted arrow indicates the shift of absorption maxima upon metal additions.

Figure S2. (A) Circular dichroism (CD) spectra of mb as isolated (thick line) and following addition of 1.0 molar equivalent of Cd(II) (thin line) and Hg(II) (gray line). (B) CD spectra of mb as isolated (thick line) and following addition of 1.0 molar equivalent of Co(II) (thin line) and Zn(II) (gray line).

Figure S3. Emission spectra of mb in aqueous solution with different excitation wavelength (nm). $\lambda_{ex} = 254, 280, 340,$ and $394 \text{ nm}$ at ambient temperature (thick lines). Arrows indicate the direction of spectrum changes upon Cd(II) (A), Co(II) (B), Fe(III) (C), Hg(II) (D), or Zn(II) (E) additions and thin lines show the spectra upon completion of changes.
Figure S4. Binding isotherm of 3.2mM CdCl2 (A), CoCl2(B), FeCl3 (C), or MnCl2(D) into 400 mM mb aqueous solution at 25°C. Curve fittings for one-site binding algorithm (B, D) or two-site binding algorithm (A, C) were used.
Figure S1.
Figure S2.
Figure S3.
Figure S4.