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NMR Studies of Methanobactin

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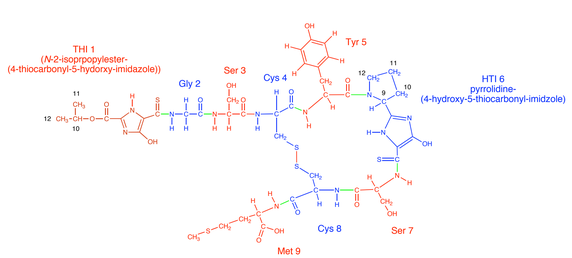
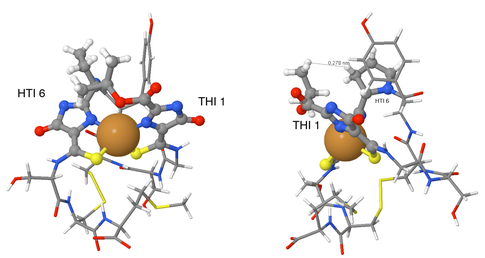
Introduction:

Methanobactin (mb) is a small copper binding molecule produced by methanotrophic bacteria. These bacteria use methane as their primary source of energy and carbon. Methanobactin can be isolated from the spent media of methanotrophs[1,2,3] and is also found within the cell associated with the copper and iron containing, particulate methane mono-oxygenase (pMMO). This enzyme, along with a soluble form of this enzyme (sMMO), catalyze the oxidation of methane to methanol.

A variety of activities are ascribed to methanobactin,[1,4,5,6] including scavenging copper from the environment, serving as a copper chaperone to pMMO, serving as an oxygen radical scavenger, mediating electron flow to pMMO, and mediating the genetic expression of pMMO.

The methanobactin that is isolated from *Methylcoccus trichosporium* OB3b contains seven amino acid residues along with two unique residues, each containing a thiocarbonyl and an imidazolate group.[7] Copper-free methanobactin, isolated under low copper levels, binds Cu(II) with high affinity and reduces it to Cu(I).[6] A crystal structure has been obtained for methanobactin after exposure to high copper levels (>1000 Cu:mb),[7] and shows one Cu bound per methanobactin and ligated by the two thiocarbonyl sulfur atoms and one of the two nitrogens from each imidazolate group. In the present study we report using NMR to elucidate the structural properties of mb at low copper levels.





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Experimental:

- The titration of methanobactin with Cu(II), as monitored by UV/Vis spectroscopy, is predominantly a two-state process.

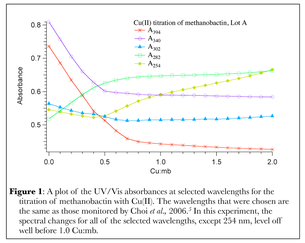

Figure 1: A plot of the UV/Vis absorbances at selected wavelengths for the titration of methanobactin with Cu(II). The wavelengths that were chosen are the same as those monitored by Choi *et al.*, 2006.[2] In this experiment, the spectral changes for all of the selected wavelengths, except 254 nm, level off well before 1.0 Cu:mb.

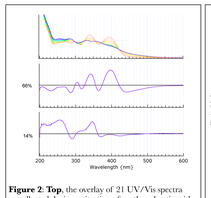

Figure 2: **Top**, the overlay of 21 UV/Vis spectra collected during a titration of methanobactin with Cu(II). The titration ranged from 0 to 2.0 Cu:mb. The methanobactin, Lot A, was dissolved to a concentration of 50 µM in 10 mM phosphate, *pH6.5*. The spectra are colored according to the rainbow from red (0.0 Cu:mb) to blue (2.0 Cu:mb). **Middle** and **bottom**, the first and second principle components obtained from a singular value decomposition of the 21 spectra. The average of the 21 spectra was subtracted from each prior to carrying out the decomposition. Together, the first and second principle components comprise 80% of the observed variations during the titration.

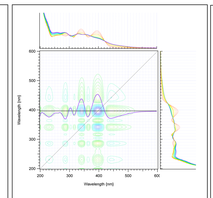

Figure 3: The synchronous component of a two-dimensional correlation analysis[8] of the 21 UV/Vis spectra collected during a titration of methanobactin with Cu(II). The titration ranged from 0 to 2.0 Cu:mb. The first principle component, which accounts for 66% of the variation during the titration, has been overlaid along the line that shows the features in the spectra that change synchronously with changes occurring at 394 nm.

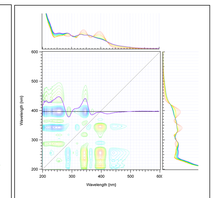

Figure 4: The asynchronous component of a two-dimensional correlation analysis of 21 UV/Vis spectra collected during a titration of methanobactin with Cu(II). The titration ranged from 0 to 2.0 Cu:mb. The second principle component, which accounts for 14% of the variation during the titration, has been overlaid along the line that shows the features in the spectra that change asynchronously with the changes that occur at 394 nm.

- The titration of methanobactin with Cu(II), as monitored by NMR spectroscopy, mirrors the results observed with UV/Vis spectroscopy.

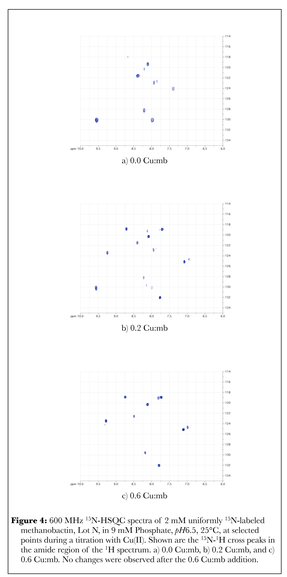

Figure 4: 600 MHz ^{15}N-HSQC spectra of 2 mM uniformly ^{15}N-labeled methanobactin, Lot N, in 9 mM Phosphate, *pH6.5*, 25°C, at selected points during a titration with Cu(II). Shown are the ^{15}N-^{1}H cross peaks in the amide region of the ^{1}H spectrum. a) 0.0 Cu:mb, b) 0.2 Cu:mb, and c) 0.6 Cu:mb. No changes were observed after the 0.6 Cu:mb addition.

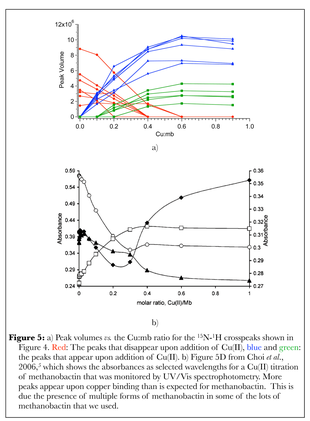

Figure 5: a) Peak volumes *vs.* the Cu:mb ratio for the ^{15}N-^{1}H crosspeaks shown in Figure 4. Red: The peaks that disappear upon addition of Cu(II), blue and green: the peaks that appear upon addition of Cu(II). b) Figure 5D from Choi *et al.*, 2006,[2] which shows the absorbances as selected wavelengths for a Cu(II) titration of methanobactin that was monitored by UV/Vis spectrophotometry. More peaks appear upon copper binding than is expected for methanobactin. This is due the presence of multiple forms of methanobactin in some of the lots of methanobactin that we used.

- The majority of the changes appear to be complete by 0.6 Cu:mb, suggesting that mb is forming a 2 to 1 complex of methanobactin to copper at low copper concentrations.

- The multiple species of methanobactin that are found in some of the lots appear to be degradation products of the methanobactin, and can be removed by performing HPLC on copper-bound methanobactin

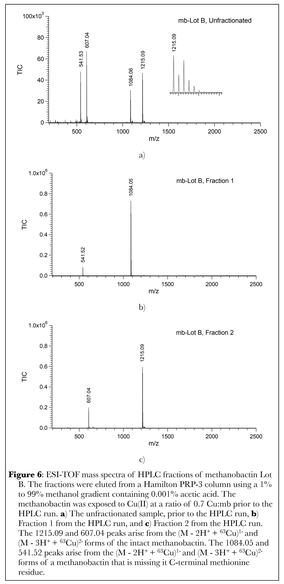

Figure 6: ESI-TOF mass spectra of HPLC fractions of methanobactin Lot B. The fractions were eluted from a Hamilton PRP-3 column using a 1% to 99% methanol gradient containing 0.001% acetic acid. The methanobactin was exposed to Cu(II) at a ratio of 0.7 Cu:mb prior to the HPLC run. **a**) The unfractionated sample, prior to the HPLC run, **b**) Fraction 1 from the HPLC run, and **c**) Fraction 2 from the HPLC run. The 1215.09 and 607.04 peaks arise from the $(M - 2H^+ + {}^{63}Cu)^{1+}$ and $(M - 3H^+ + {}^{63}Cu)^{2+}$ forms of the intact methanobactin. The 1084.05 and 541.52 peaks arise from the $(M - 2H^+ + {}^{63}Cu)^{1+}$ and $(M - 3H^+ + {}^{63}Cu)^{2+}$ forms of a methanobactin that is missing it C-terminal methionine residue.

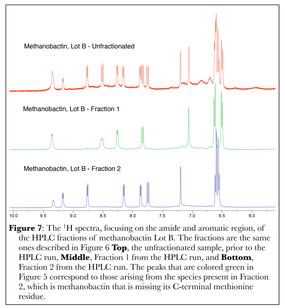

Figure 7: The ^{1}H spectra, focusing on the amide and aromatic region, of the **HPLC** fractions of methanobactin Lot B. The fractions are the same ones described in Figure 6 **Top**, the unfractionated sample, prior to the HPLC run, **Middle**, Fraction 1 from the HPLC run, and **Bottom**, Fraction 2 from the HPLC run. The peaks that are colored green in Figure 5 correspond to those arising from the species present in Fraction 2, which is methanobactin that is missing its C-terminal methionine residue.

- ESI-TOF mass spectroscopy shows that one of the species has the mass expected for a 1 to 1 methanobactin to copper complex.
- The other has a mass that is consistent with a 1 to 1 methanobactin to copper complex that forms with a methanobactin that is missing is C-Terminal methionine residue. This conclusion has been corroborated by NMR spectroscopy.

- In theses and other experiments we have found no mass spectroscopy evidence to support the formation of a 2 to 1 methanobactin to copper complex during the titration of methanobactin with Cu(II).

- Using a combination of COSY, TOCSY and ROESY experiments, we have made proton assignments for methanobactin that are consistent with its reported structure, with one notable exception:

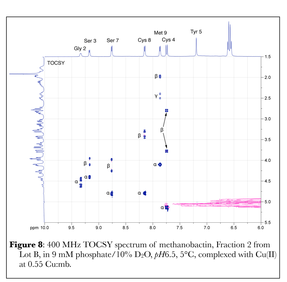

Figure 8: 400 MHz TOCSY spectrum of methanobactin, Fraction 2 from Lot B, in 9 mM phosphate/10% D_2O, *pH6.5*, 5°C, complexed with Cu(II) at 0.55 Cu:mb.

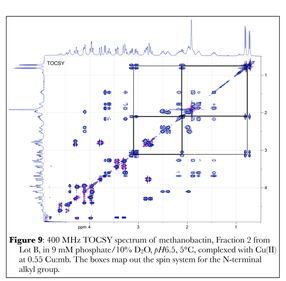

Figure 9: 400 MHz TOCSY spectrum of methanobactin, Fraction 2 from Lot B, in 9 mM phosphate/10% D_2O, *pH6.5*, 5°C, complexed with Cu(II) at 0.55 Cu:mb. The boxes map out the spin system for the N-terminal alkyl group.

- The exception is the assignment for the N-terminal alkyl group. The published spectrum has this as an isopropyl ester, the NMR evidence (Figure 9), suggests an isobutyl ketone.

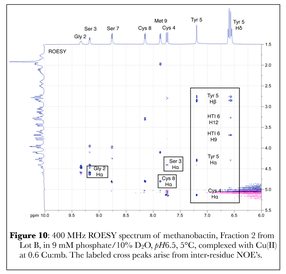

Figure 10: 400 MHz ROESY spectrum of methanobactin, Fraction 2 from Lot B, in 9 mM phosphate/10% D_2O, *pH6.5*, 5°C, complexed with Cu(II) at 0.6 Cu:mb. The labeled cross peaks arise from inter-residue NOE's.

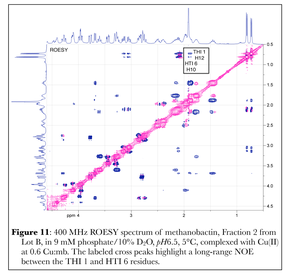

Figure 11: 400 MHz ROESY spectrum of methanobactin, Fraction 2 from Lot B, in 9 mM phosphate/10% D_2O, *pH6.5*, 5°C, complexed with Cu(II) at 0.6 Cu:mb. The labeled cross peaks highlight a long-range NOE between the THI 1 and HTI 6 residues.

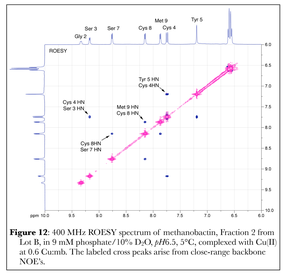

Figure 12: 400 MHz ROESY spectrum of methanobactin, Fraction 2 from Lot B, in 9 mM phosphate/10% D_2O, *pH6.5*, 5°C, complexed with Cu(II) at 0.6 Cu:mb. The labeled cross peaks arise from close-range backbone NOE's.

- The AMX spin system rising up from the Tyr H_N proton is conspicuously missing from the TOCSY spectrum (Figure 8). The assignments for the protons in this system were made based on their NOE's in the ROESY spectrum (Figure 10).

- The long-range NOE observed between one of the methyl groups of the THI 1 residue and one of the methylene groups of the pyrrolidine ring of the HTI 6 residue (Figure 11), suggests a solution structure that is similar to the the crystal structure, where the distance between these protons approaches 0.278 nm (see structure shown to the left).

Conclusions:

- When the titration of methanobactin with Cu(II) is monitored by NMR spectroscopy, a predominantly two-state process is observed. This is in good agreement with what is is observed using other spectroscopic methods.
- The changes in the spectra appear to level off at around 0.6 Cu:mb, suggesting a dimer of methanobactin forms, which shares a single Cu(I) ion.
- When Cu(II) is bound and reduced by methanobactin, more than the expected number of amide resonances appear. This can be attributed to the presence of degraded species of methanobactin, and in particular a species that is missing its C-terminal methionine residue, which are still able to bind Cu(II).
- The ^{1}H resonance assignments for methanobactin have been made and agree, for the most part, with the published structure. A notable exception is the N-terminal alkyl group, which appears by NMR to be an isobutyl ketone instead of an isopropyl ester. In order to make this change, other changes would need to be made to the methanobactin structure to remain consistent with the mass spectroscopy data. Also, there appears to be little or no ^{3}J-coupling between the Tyr 5 H_N and H_α protons.
- A long-range NOE that appears in the ROESY spectrum suggests the solution structure that forms upon copper binding at low copper concentrations is similar to the crystal structure that forms at high copper concentrations. This, along with the mass spectroscopy data, support the formation of a 1 to 1 complex of methanobactin and copper. This conclusion directly contradicts the conclusion formed from the titration data.

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- NSF-MRI Grant CHE 0619296 to UW-Eau Claire for the purchase of an Agilent 6210 ESI-TOF LC/MS