The Structure of Methanobactin SB2 May Help to Define a Whole New Class of Peptide-derived Metal-binding Molecules

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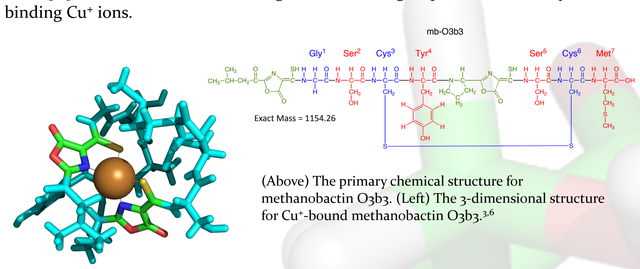
Introduction

Methanobactin is peptide-derived, copper-binding molecule that is produced by methanotrophic bacteria.[1-8]

- Methanotrophs use methane from the atmospheric as their primary carbon and energy source and the first step in metabolizing methane is oxidizing it to methanol.
- The enzyme that converts methane to methanol is called *particulate methane mono-oxygenase* (pMMO). pMMO requires copper ions and methanotrophs synthesize methanobactin for the purpose of scavenging copper ions from the environment to meet this needs for this enzyme.
- The methanobactin produced by the methanotroph *Methylosinus trichosporium O3b3* (mb-O3b3) is the only methanobactin that has been well characterized so far.
- mb-O3b3 binds Cu^{2+} ions, reduces them to Cu^{+} ions, and stabilizes the resulting Cu^{+} ions using a mechanism that is not well understood.
- mb-O3b3 is also able to bind and reduce other metal ions as well.
- mb-O3b3 contains two oxazolone rings and two thiol groups that are directly involved in binding Cu^{+} ions.



(Above) The primary chemical structure for methanobactin O3b3. (Left) The 3-dimensional structure for Cu^{+}-bound methanobactin O3b3.[3,6]

Here, we report our results for determining the chemical structure of a new form of methanobactin, methanobactin SB2 (mb-SB2), which is produced by the methanotroph *Methylocystis daltona SB2*, and comparing its structure to the previously determined one for mb-O3b3.

- The comparison is proving valuable for highlighting the features that are important to a functioning methanobactin molecule.
- These two methanobactins share a couple of unusual features which suggest that mb-SB2 and mb-O3b3 may represent members of a whole new class of molecules that have evolved from simple peptides into powerful agents for binding, reducing and stabilizing metal ions in aqueous environments.

Methods Used in Our Studies

Ultraviolet/Visible (UV/Vis) Spectrophotometry

- Ultraviolet and visible light are can be absorbed by molecules. This is what produces the colors we seen in the world around us.
- The aromatic rings in mb-O3b3 and mb-Sb2 have a characteristic absorption that can be used to monitor these rings.

Nuclear Magnetic Resonance (NMR) Spectroscopy

- Some of the atomic nuclei in molecules are magnetic.
- When placed in a strong magnetic field, these nuclei will interact with the applied magnetic field.
- Examples of magnetic nuclei found in mb-O3b3 include the ^{1}H isotope of hydrogen, the ^{13}C isotope of carbon, the ^{15}N isotope of nitrogen.
- This interaction can be probed with radio waves.
  - Typically, the data are displayed as a 1-dimensional spectrum, which plots the intensity of absorbance against the frequency (ppm) at which a nucleus absorbs a radio wave.
  - Each magnetic atomic nucleus in a molecule will be represented by a peak in an NMR spectrum.
  - The resonance frequency for an atomic nucleus is influenced by its local environment.
- Magnetic nuclei can also "talk" to one another when placed in a magnetic field
  - NMR spectroscopy can be used to listen in on these conversations and to discover who is talking to whom.
  - These conversations provide a wealth of information about the structure of a molecule.

Mass Spectroscopy

- A mass spectrometer is able to measure the mass-to-charge ratio (m/z) of charged molecules.
- Our Time-of-Flight is able to measure mass to charge ratios to accuracy of 2 parts per million!

X-ray Photoelectron Spectroscopy (XPS)

- When X-rays interact with a sample they can eject high energy electron by a process called the photoelectric effect.
- The kinetic energy of the electrons can be measured and are characteristic of the atoms they were ejected from.
  - This method can be used to measure the presence and electronic environment of specific atoms in a sample.

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- Dr. Alan DiSpirito (Iowa State University) and Dr. Jeremy Semrau (University of Michigan) for supplying us with the methanobactin samples
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- Dr. Scott Hartsel for sharing mb-SB2 copper binding results
- Dr. David Lewis for helpful discussions.
- Ms. Heidi Mulheron for contributions to the procedures used in the hydrolysis experiments

Results Summary

Our initial investigations of mb-SB2 involved ultraviolet/visible light (UV/Vis) spectroscopy to observe the light absorbing rings in mb-SB2. In mb-O3b3, two oxazolone rings are responsible for the absorbance peaks at wavelengths of 340 nm and 394 nm.

- mb-SB2 has a very similar absorption spectrum to mb-O3b3, suggesting it too contains a pair of oxazolone rings

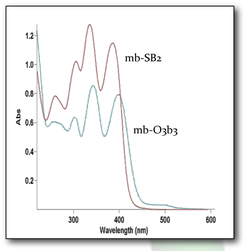


Figure 1. UV/Visible Spectrum Comparison of Cu-bound SB2 and O3b3. Samples of mb-SB2 and mb-O3b3 dissolved in 10 mM phosphate buffer. Here, the chromophoric or color-producing rings of SB2 and O3b3 are highlighted for wavelengths between 220-600 nm. Notice how the curves seem to match and track each other.

- ...BUT, mass spectrometry (MS) gave a mass approximately 20% lighter than O3b3 (Figure 2). mb-O3b3 has a mass of 1154.26, while the mass spectrum for mb-SB2 indicates an uncharged mass of 851.19!

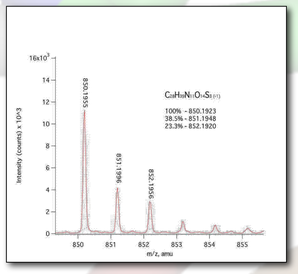


Figure 2. Mass Spectrum of Cu^{+}-free mb-SB2 (-1H^{+}). In order to analyze samples of Cu^{+}-free SB2, we dissolved the sample in 10 mM phosphate buffer and purified the Cu^{+}-free mb-SB2 using High-Pressure Liquid Chromotography (HPLC) and eluting the fractions with a .01-99.9% gradient of methanol in water. The purified Cu^{+}-free fraction was lyophilized and and used for both NMR spectroscopy and mass spectrometry. Shown above is the mass spectrum of Cu^{+}-free SB2 in its -1 charged state (having lost 1 hydrogen ion). The smaller peaks to the right of the the largest peak represent the presences of heavier isotopes for some of the atoms that make up mb-SB2. The separation of each peak represent the mass of one neutron.

Together, the UV/Vis and mass spectrometry data suggest that while the mb-SB2 appears to have similar light absorbing rings as the oxazolone rings in mb-O3b3, there must also be some major differences in its structure to account for the substantial difference in mass. We then turned to NMR spectroscopy to help us elucidate these differences. As described in the Methods section, NMR spectroscopy allows us to probe individual atomic nuclei in a molecule to find out things about these atoms and their neighbors. This allows should us to infer an chemical structure for a molecule.

- Figure 3 shows both a 1-dimensional ^{1}H-NMR spectrum of Cu^{+}-bound SB2, in which each peak in the spectrum represents a different group of hydrogen atoms in the molecule. Also shown is a 2-dimensional ^{1}H-^{1}H-COSY spectrum, which shows, as cross peaks, which hydrogen atoms in the molecule are within 2-bonds of each other.

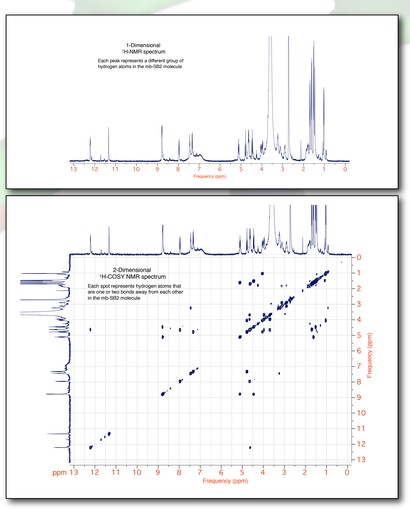


Figure 3. (top) A 1-D ^{1}H spectrum of mb-SBW, (bottom) a 2D ^{1}H-^{1}H-COSY NMR of Cu-bound SB2. In the COSY spectrum you can see the 1-D ^{1}H spectrum both at the top and along the left hand side. The spots that appear off of the diagonal tell us which of the hydrogen atoms that give rise to the peaks in the 1D spectrum are with in one or two bonds of one other in the molecule.

There are a collection of different NMR experiments that go by a range of different acronyms, which can be used to map out a structure for a molecule. Figure 4 shows the results of some of the experiments that we used on mb-SB2. At the top of the figure is a proposed structure that is consistent with the NMR and UV/Vis results.

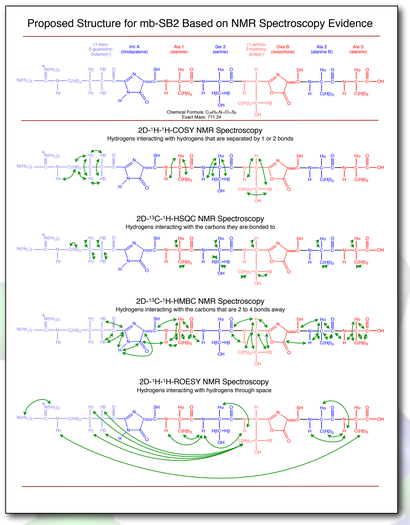
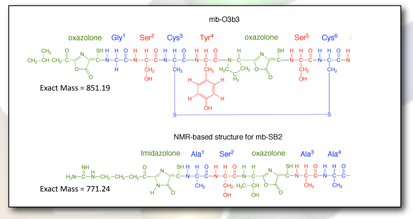


Figure 4. NMR mapping of the mb-SB2 primary structure. Using the results of various NMR experiments, we derived a proposed primary structure for mb-SB2.

- The NMR spectroscopy studies reveal that mb-SB2, like mb-O3b3, contains amino acid residues. in addition to the light absorbing, aromatic rings. There identities, however, are quite different.



- NMR evidence also suggested one of the UV/Vis absorbing rings is an imidazolone ring instead of an oxazolone ring.
  - Extensive acid catalyzed hydrolysis of mb-SB2 by and the study of the hydrolyzed fragments by UV/Vis spectrophotometry, liquid chromatography, mass spectroscopy, and NMR spectroscopy, demonstrated that one of the rings in mb-SB2 behaves like an imidazolone instead of an oxazolone ring.
  - We also did 1D- and 2D-^{15}N-NMR experiments, which confirmed that mb-SB2 has the correct number of nitrogen atoms for a structure containing an imidazolone ring (Figure 5)

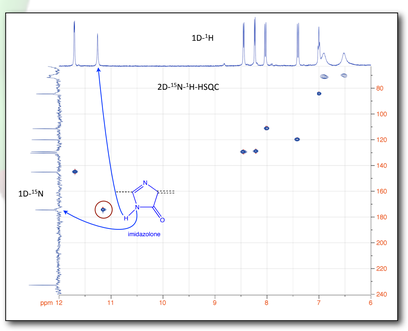


Figure 5. 1-Dimensional and 2-dimensional ^{1}H and ^{15}N NMR spectra of mb-SB2.

...BUT, the structure that we determined by NMR (Figure 4) has a problem, at a calculated mass of 771.24 atomic mass units, it is 80 amu short of the observed 851.20 amu for mb-SB2 (see Figure 2)!

- Possible candidates that we considered for the missing mass included:
  - A phenyl group, but this should have been obvious by NMR spectroscopy.
  - Selenium, an element sometimes associated with peptides, but this should have given a unique isotopic pattern in the mass spectrum, which was not observed.
  - A phosphate group is reasonable candidate; however, we did not observe any phosphorous atoms in mb-SB2 using ^{31}P-NMR.
- Our remaining candidate was a sulfate group.
  - Our original attempt look for a sulfate group consisted of attempting to hydrolyze SB2 in the presence of $BaCl_2$ and to observe whether a $BaSO_4$ precipitate formed. However, our conclusion after multiple attempts was that a significant portion of mb-SB2 is stable to acid hydrolysis, and that detectable quantities of $BaSO_4$ may not form.

We next tried X-ray photoelectron spectroscopy (see Methods section), which is able to identify elements based on the kinetic energy of electrons that are ejected when the sample is irradiated with X-rays. Figure 6 shows the sulfur region of the XPS spectrum for mb-SB2.

- The XPS spectrum clearly shows the presence of a sulphate group in mb-SB2. It also confirms the presence of the two sulfide's, which are also found in mb-O3b3.

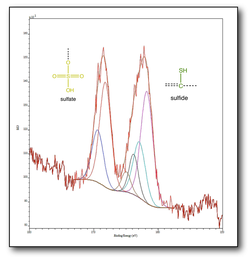
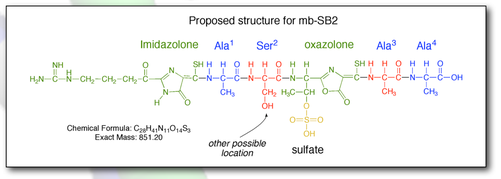


Figure 6. XPS demonstrating the presence of sulfate. Here, sulfate from Cu^{+}-free SB2 ejects electrons with a higher binding energy (169.3 eV) than sulfides. This spectrum both demonstrates the presence of sulfate in mb-SB2 and confirms the presence of the the two sulfide groups, which, like the two those found in mb-O3b3, are involved in binding the Cu^{+} ions.

We are still uncertain where the sulfate is located on the mb-SB2 molecule, however, one of the two hydroxyl groups (-OH) are the most likely locations.

- Our current proposed structure is shown below. The calculated mass for this structure is 851.20 amu, in good agreement with our measured value of 851.19 amu

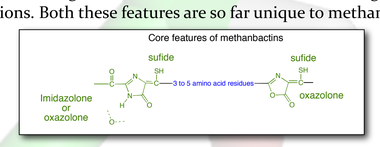


Discussion

A comparison of our proposed structure for mb-SB2 with the previously determined structure for mb-O3b3 reveals some interesting similarities and differences.

Similarities

- mb-SB2, like mb-O3b3, is capable of binding Cu^{2+} ions and reducing them to Cu^{+} in an aqueous environment.
- Both molecules appear to be derived from a peptide of amino acids.
- Both contain similar five-member rings in associated with a sulfide group, which together, are believed to serve as the binding site for metal ions. Both these features are so far unique to methanobactins.



Differences

- The numbers and identities of the amino acids used are quite different.
- mb-SB2 contains an imidazolone ring in place of one of the oxazolone rings found in mb-O3b3
- mb-SB2 contains a sulfate group, which is very unusual. mb-SB2 may represent the first example of this kind of sulfate group in a bacterial derived peptide.[9]

Together, our findings suggest that mb-O3b3 and mb-SB2 may represent the first discovered examples from a whole new class of peptide derived molecules that have evolved to bind, reduce, and stabilize copper, as well as other metal ions.

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