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The Copper Chelator Methanobactin from *Methylosinus trichosporium* OB3b Binds Copper(I)

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Methanobactin is a small siderophore-like molecule proposed to function in copper sequestration and handling by methanotrophs, methane-oxidizing bacteria that play a key role in the global carbon cycle.[1] Copper is critical to methanotroph metabolism, regulating expression of two methane monooxygenase (MMO) systems,[2,3] soluble MMO (sMMO)[4] and particulate MMO (pMMO)[5] as well as of other metabolic enzymes.[6] In addition, copper is a pMMO cofactor[7–9] and stimulates the formation of intracytoplasmic membranes that house pMMO.[3,10] Methanobactin may not function solely in copper uptake, but could also play a more direct role in pMMO loading and activity. Originally referred to as a copper-binding compound (CBC), methanobactin was first detected a decade ago,[11–13] but difficulties with degradation precluded structural characterization until recently.[14,15] The sequence of methanobactin from *Methylosinus trichosporium* OB3b is *N*-2-isopropylester--(4-thionyl-5-hydroxyimidazolate)-Gly[1]-Ser[2]-Cys[3]-Tyr[4]-pyrrolidine-(4-hydroxy-5-thionylimidazolate)-Ser[5]-Cys[6]-Met[7], and the crystal structure shows a single copper ion coordinated by an N_2S_2 donor set (Scheme 1).[14,15] On the basis of its structural similarity to iron siderophores,[16] methanobactin may have antibacterial properties.[14] Additional practical applications for methanobactin potentially include its use in the semiconductor industry to remove copper from wastewater.[17] Despite its potential broad importance, key information about methanobactin, such as the oxidation state of the chelated copper ion, is lacking. Here we show by electron paramagnetic resonance (EPR) and X-ray absorption (XAS) spectroscopies that methanobactin binds copper in the 1+ oxidation state.

Spent media from *M. trichosporium* OB3b was incubated with $CuCl_2$, and methanobactin was isolated and purified by protocols similar to those described by Graham and co-workers.[12,15] Analysis of the final product by MALDI-MS or ESI-MS gave a single peak at 1215 Da, corresponding to an 1153 Da methanobactin molecule which has lost one or two hydrogen atoms and bound one copper atom. Anaerobic incubation of spent media with CuCl also gave a peak at 1215 Da (Figure S1), indicating that apo methanobactin can be fully loaded with one copper ion by treatment with either Cu(II) or Cu(I).

The X-band EPR spectrum of Cu(II)-loaded methanobactin (Figure 1, blue trace) shows a weak, poorly resolved signal with $g_{||} = 2.32(2)$, $g_{\perp} = 2.07(1)$, and $A_{||} = 420(20)$ MHz (130 G) (all parameters determined by spectral simulation). A similar sized peptide from *M.*

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Supporting Information Available: A table of EXAFS fitting analysis, ESI-MS data, and details of methanobactin purification and spectroscopic data collection. This material is available free of charge via the Internet at http://pubs.acs.org.

trichosporium OB3b, denoted CBC-L_1, gave EPR parameters of $g_{||}$ = 2.42, $g_{\perp}$ = 2.087, and $A_{||}$ 128 G,[13] and together with EPR data on a less pure preparation from *Methylococcus capsulatus* (Bath)[11] suggested the presence of Cu(II). These $g_{||}$ and $A_{||}$ values are more characteristic of Cu(II) with N_xO_{4-x} (x = 0, 1, 2) coordination than of Cu(II) with sulfur ligation, which typically exhibits much smaller $g_{||}$ values.[18] No EPR quantitation was reported for the *M. trichosporium* OB3b CBC-L_1. The spin Hamiltonian parameters and low intensity of the EPR signal in all of these cases suggest that the signal arises from adventitious Cu(II), however. To determine if our methanobactin samples contained EPR-silent Cu(I), we added the strong oxidant nitric acid, which generated an intense Cu(II) signal. The EPR parameters for this signal (Figure 1, red trace) are $g_{||}$ = 2.40(1), $g_{\perp}$ = 2.07(1), and $A_{||}$ = 430(10) MHz (130 G). These $g_{||}$ and $A_{||}$ values are characteristic of Cu(II) with an O_4 donor set18 as expected for methanobactin-bound Cu(I) that is released and oxidized by nitric acid. Methanobactin is known to degrade and lose copper at low pH.[15] The quantity of Cu(II) present after nitric acid treatment, as measured by double integration using $[CuEDTA]^{2-}$ as a standard, increased significantly, by a factor of 6.7 ± 0.7 (measurement repeated on triplicate samples). Metal analysis by atomic absorption spectroscopy indicates the presence of 1.09 ± 0.21 copper ions per methanobactin molecule. These data indicate that copper is bound to methanobactin as Cu (I) with the presence of a small amount of adventitiously bound Cu(II). This finding is consistent with previously reported X-ray photoelectron spectroscopic (XPS) data interpreted as evidence for Cu(I). In that study, however, appropriate standards containing Cu(I) or Cu(II) bound to N,S ligands were not examined by XPS.[14]

To further investigate the copper oxidation state in methanobactin, we collected Cu K edge XAS data on Cu(II)-loaded samples. The XANES spectrum (Figure 2, top) clearly indicates the presence of Cu(I), as shown by the presence of a 1s → 4p transition at 8985 eV and edge features resembling four coordinate cuprous models.[19] The lack of a 1s → 3d transition at 8980 eV, characteristic of Cu(II), further shows there is no appreciable Cu(II).[19] Copper EXAFS data (Figure 2, bottom) were best fit using a multiple scattering model generated from the crystallographic parameters.[14] Long-range scattering, reminiscent of imidazole scattering patterns from coordinated histidines,[20] includes an additional carbon scattering environment at 4.16 Å. The data can also be reasonably fit with single scattering models, however (Table S1). The excellent agreement between the simulations with two Cu–N interactions (2.03 and 2.05 Å) and two Cu–S interactions (2.34 and 2.40 Å) in a combined multiple scattering model system reflects a copper coordination environment nearly identical to that observed in the crystal structure.

Our data establish definitively that *M. trichosporium* OB3b methanobactin binds Cu(I) despite the fact that loading can be accomplished by the addition of Cu(II). Thus, methanobactin itself probably reduces Cu(II) to Cu(I). A reasonable scenario is that Cu(II) reduction to Cu(I) is coupled to disulfide bond formation in methanobactin. How the Cu(I)-bound methanobactin is translocated into the cell and how the Cu(I) is distributed to pMMO and other targets remain to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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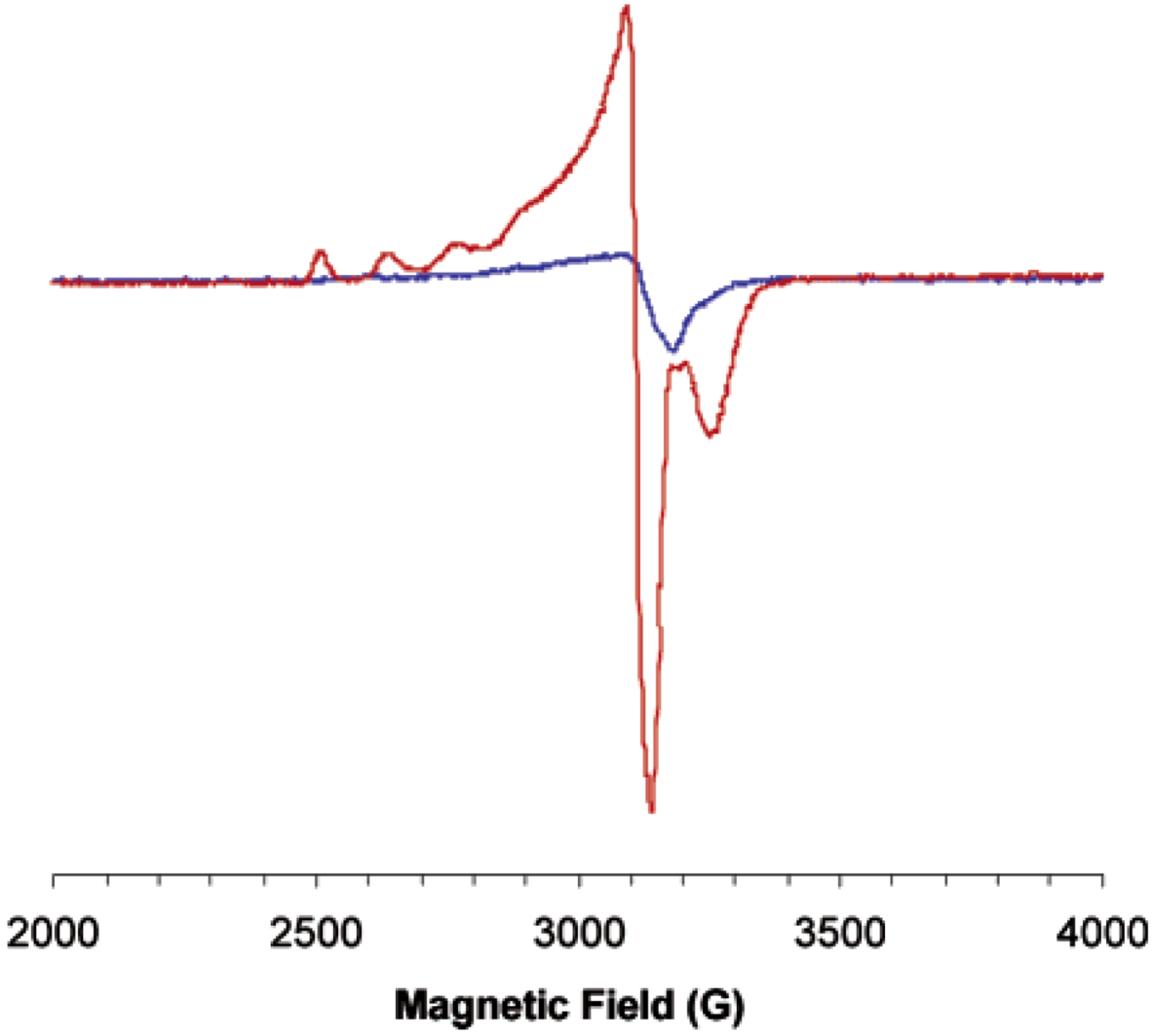


Figure 1.
Representative EPR spectra of Cu(II)-loaded methanobactin (2.2 mM) from *Methylosinus trichosporium* OB3b before (blue, 0.39 mM Cu(II)) and after (red, 3.0 mM Cu(II)) the addition of 2.5% concentrated nitric acid. Experimental conditions: temperature, 77 K; microwave frequency, 9.31 GHz; microwave power, 10 mW; field modulation amplitude, 5 G; time constant, 160 ms; scan time, 2 min.

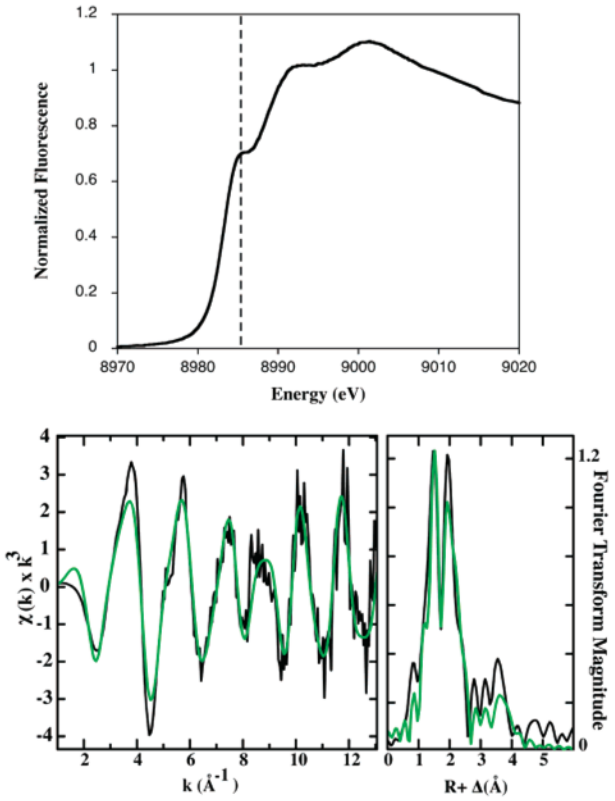
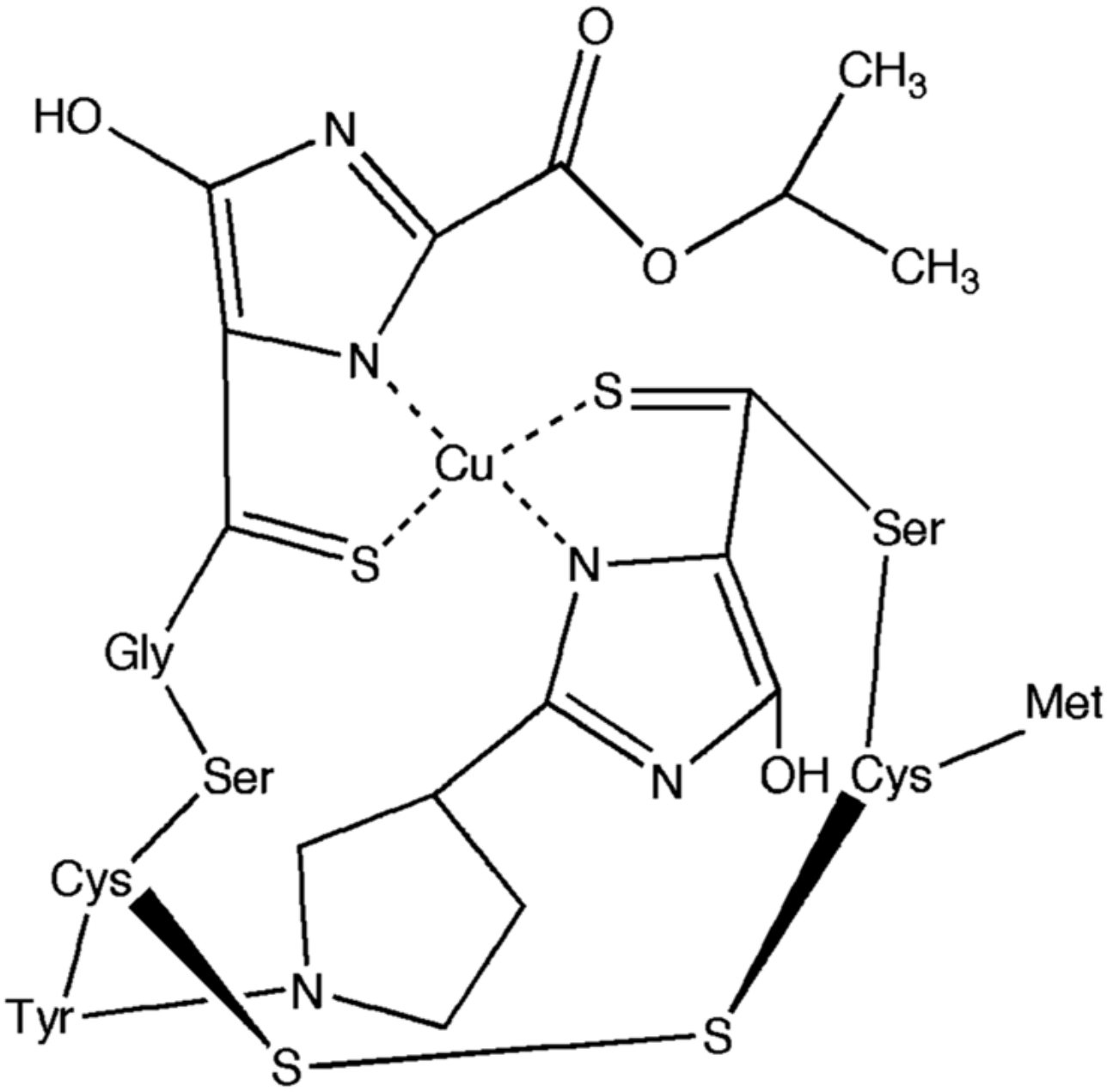


Figure 2.
XAS analysis of Cu(II)-loaded methanobactin. Top: Cu XANES spectrum. Dashed line identifies the 1s → 4p transition at 8985 eV. Bottom: EXAFS (left) and its Fourier transform (right). Raw data are shown in black with the best fit superimposed in green.



Scheme 1.