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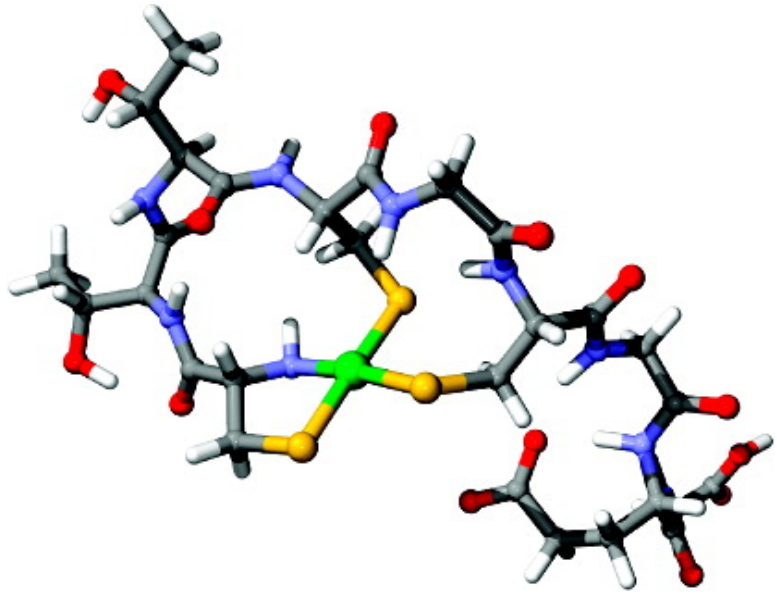

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A High-Affinity Metal-Binding Peptide from *Escherichia coli* HypB

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The biosynthesis of metalloenzymes often depends on dedicated metallochaperones to deliver the metals to the functional sites.[1,2] One such system is the maturation of the bimetallic catalytic center in the [NiFe] hydrogenase, which contains nickel and iron ions bound to the protein by cysteine ligands.[3] The incorporation of nickel into the hydrogenase enzyme precursor protein requires the activity of several accessory proteins including HypB,[4,5] but how this event occurs is not clear. *Escherichia coli* HypB binds metal at two sites: one that involves the cysteines of the N-terminal CXXCGC motif and demonstrates a very high-affinity for nickel, the other a weaker site in the C-terminal domain.[6] In an effort to further our understanding of how HypB contributes to hydrogenase biosynthesis and to provide information about nickel-binding sites in biological systems, the high-affinity nickel site of HypB was isolated and characterized in detail.

Previous X-ray absorption spectroscopy (XAS) of HypB loaded with stoichiometric Ni(II) revealed a square planar geometry with three thiolate ligands and one N/O ligand.[7] Mutagenesis studies suggested that the three N-terminal cysteines of the protein bind to nickel,[6] but the identity of the fourth ligand was unknown. One feasible hypothesis is that the complete high-affinity metal-binding site is contained in the N-terminal segment of the protein, and this possibility was tested by appending the first 10 amino acids of HypB (MCTTCGCGEG) to the N-terminus of human ubiquitin. Ubiquitin, which has been previously used as a scaffold for metal-binding tags,[8] was chosen due to its lack of cysteines and metal-binding properties as well as its solubility. The resulting fusion protein was named B9ubiquitin because of the loss of the initial methionine upon expression in *E. coli*, as observed with wild-type HypB.[6]

The purified protein was rendered apo under anaerobic conditions, and the free thiol content was verified with DTNB (see Supporting Information (SI)). Nickel titrations performed anaerobically on B9ubiquitin revealed a linear increase in absorbance at 315 nm that saturated at 1 equiv of metal (Figure S1, inset), with a similar spectrum to that observed for HypB loaded with stoichiometric nickel (Figure S1). HPLC-PAR analysis[9] was used to confirm that 1 equiv of nickel remained bound to the protein following gel filtration chromatography. The participation of the cysteines as ligands to the nickel ion was verified by titrating holo-B9ubiquitin with the thiol-reactive reagent PMB in the presence of the metallochromic indicator PAR (Figure S2). Parallel experiments performed with ubiquitin alone did not produce evidence of nickel binding (data not shown). Metal release experiments suggested that the nickel ion was bound tightly to B9ubiquitin (Figure S3). This result was confirmed by competitive nickel-binding experiments in the presence of EGTA which revealed a K_d of (4.4 $\pm$ 0.8) $\times$ 10^{-13} M (Figure 1), similar to that of full-length HypB ((1.3 $\pm$ 0.2) $\times$ 10^{-13} M).[6]

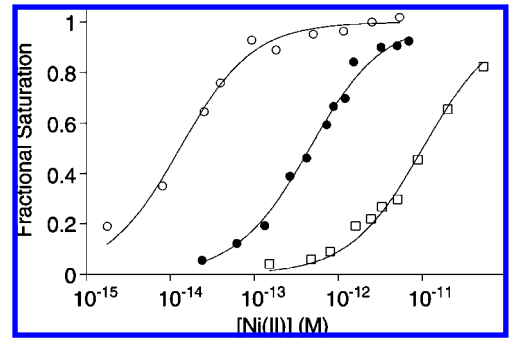

Figure 1. Nickel titration experiments with 10 μM B9ubiquitin (●) or B9E8A (○) and 5 mM EGTA, or 5 μM B9peptide (□) and 1 mM EGTA. Data from several experiments were fit to a Langmuir isotherm to calculate average K_d's of (4.4 $\pm$ 0.8) $\times$ 10^{-13}, (2 $\pm$ 1) $\times$ 10^{-14}, and (2 $\pm$ 1) $\times$ 10^{-11} M respectively.

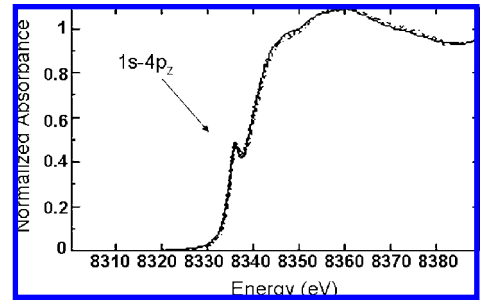

Figure 2. Ni(II) near-edges. The spectra of HypB (—),[7] B9ubiquitin (---), or the B9peptide (-•-) loaded with 1.0 equiv of Ni(II) are overlaid, normalized to the edge jump. The arrow indicates the 1s-4p_z transition that is characteristic of Ni(II) square-planar complexes.

XAS of Ni(II)-B9ubiqutin produces a strikingly similar near-edge spectrum to that of nickel-loaded HypB,[7] displaying a 1s-4p_z pre-edge peak at 8334 eV that is typical of square planar Ni(II) complexes (Figure 2).[10] The EXAFS spectra are best fit to a S_3N/O coordination sphere (Figure S4 and Table S3).[11] The similar electronic absorption and XAS spectra of nickel-loaded B9ubiquitin compared to those of HypB, coupled with tight nickel binding, indicate that this nickel site is contained in the N-terminal peptide of HypB. Furthermore, they confirm the assignment of the three cysteines within the CXXCGC motif as ligands and demonstrate that the nitrogen or oxygen donor also originates from the N-terminal 9 amino acids of HypB.

Peptide maquettes are often valuable tools because they are small molecule representations of the cofactor-containing sites of metalloproteins.[12,13] To determine if a HypB maquette can reproduce the appropriate chemical properties of the high-affinity nickel site out of the context of the full protein environment, the B9peptide CTTCGCGEGW was prepared by using solid-phase peptide syn-

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thesis (see SI). The tryptophan residue was appended to the C-terminal end of the HypB sequence to aid in detection and quantification. Direct nickel titrations of the B9peptide revealed quantitative, stoichiometric nickel binding (data not shown) and produced similar electronic absorption and XAS spectra as observed for the proteins (Figures 2, S1, and S4). Furthermore, competition with 1 mM EGTA revealed a K_d of $(2 \pm 1) \times 10^{-11}$ M (Figure 1). Together, these results are consistent with a square-planar, S_3O/N site that binds nickel with high affinity, albeit slightly weaker than when attached to a larger protein molecule.

Full characterization of this nickel-binding motif required the identification of the N/O ligand. One reasonable option is the glutamate that is two residues downstream of the cysteine motif. To test this possibility, site-directed mutagenesis was used to prepare the E8A mutation in B9ubiquitin, B9E8A. Upon addition of nickel to the apo, reduced protein, the mutant exhibited quantitative nickel binding with a similar electronic absorption spectrum as that of the other B9 constructs as well as a nickel affinity slightly tighter than that for the wild-type protein ($(2 \pm 1) \times 10^{-14}$ M, Figures 1, S1, S3). These results indicate that the nickel site is intact in the B9E8A mutant and that Glu8 does not coordinate to the nickel ion.

A second potential O/N ligand is the free amine at the protein N-terminus, which serves as a metal ligand in other proteins.[14−16] The N-terminus of B9ubiqitin was modified by taking advantage of the cysteine residue at the end and using *S*-(4-nitrophenyl)thioacetate to introduce an *N*-acyl functional group. A single modification was detected by using MALDI-MS, and proteolytic digestion or iodoacetamide modification followed by MS verified that the final modification was at the N-terminal amino group and not on a cysteine (see SI for modification reaction and characterization). In contrast to all other B9 constructs, the addition of nickel to modified B9ubiquitin, AcB9, resulted in a shifted electronic absorption spectrum with a maximum at 262 nm (Figure S1). Furthermore, this nickel binding was not strong enough to compete with EGTA and was comparable to that of 10 mM glycine, a weaker nickel competitor (data not shown).[17] These results demonstrate that the modification causes a change in the coordination sphere of the nickel site and suggest that the N-terminal amine is the fourth metal-binding ligand.

The electronic absorption spectroscopy and XAS of the B9 constructs reveal that the entire high-affinity nickel site of HypB is in the N-terminal fragment of the protein and that this peptide is sufficient for tight nickel binding. Mutagenesis and chemical modification indicate that the square-planar complex is composed of the N-terminal amine in addition to three cysteines. DFT calculations of this Ni(II) complex produced bond lengths that are in excellent agreement with those measured by XAS (Figure 3). Furthermore, the calculations suggest that the N-terminal amine is deprotonated in the Ni(II)−B9 peptide complex because calculations with the protonated form resulted in a longer Ni−N bond length. This type of N-terminal complex is similar to two other known Ni(II) proteins, the catalytic center of nickel superoxide dismutase[15,16] and the amino terminal Cu(II)- and Ni(II)-binding (ATCUN) motif.[14] The ATCUN site is found in some serum albumins, as well as a variety of other proteins, where it contributes to metal transport. In analogy, the N-terminal nickel site of HypB, which is required for *E. coli* hydrogenase biosynthesis under standard

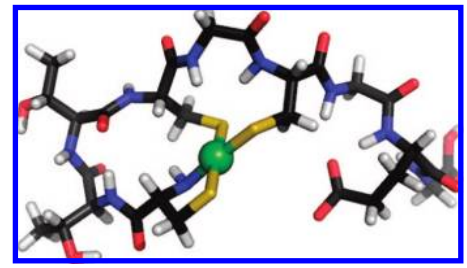

Figure 3. Molecular modeling of Ni(II)-B9peptide. The calculated bond lengths of the energy-minimized structure are (Ni−N) 1.922 Å and (Ni−S) 2.235, 2.270, and 2.282 Å.

growth conditions,[7] could also be involved in metal transport. The CXXCGC motif is not conserved in all HypB homologues, and given the tight nickel affinity, it is plausible that it serves as the source of nickel for hydrogenase under distinctive growth conditions such as nickel limited. Furthermore, a recent study demonstrated that metal release from this site is influenced by another accessory protein, SlyD.[18] How this site changes in the context of the complete nickel insertion complex and the steps in nickel delivery to the hydrogenase enzyme are the subjects of future investigations.

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Supporting Information Available: Details for protein expression, purification, modification, B9peptide synthesis, PMB titration, metal analysis, nickel titrations, metal release assays, verification of modification by MALDI-MS, fits for the EXAFS, and DFT calculations. This material is available free of charge via the Internet at http://pubs.acs.org.

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