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Published on Web 08/16/2006

Thermodynamic and Kinetic Aspects of Metal Binding to the Histidine-rich Protein, Hpn

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Helicobacter pylori is a Gram-negative bacterium that colonizes the stomach and causes gastritis and peptic ulcerations.[1] Its adaption to this hostile environment is linked to the production of abundant urease, a dinuclear nickel-containing metalloenzyme catalyzing the hydrolysis of urea to ammonia and carbamate for neutralization of gastric acid, which is critical for survival under low pH conditions.[2,3] Therefore, a constant supply of nickel ions is required for the synthesis and activity of urease (and hydrogenase) by *H. pylori*. However, the accumulation of free nickel ions should be avoided due to its inhibition of growth, competition with other essential metal ions, and generation of reactive oxygen species.[4]

H. pylori has a small histidine-rich cytoplasmic protein, Hpn (~2% of total protein synthesized), that has been proposed to play a role in nickel storage.[5] Hpn-deficient *H. pylori* mutants are more sensitive to excess Ni^{2+} and Bi^{3+} (as ranitidine bismuth citrate) than the wild type,[6] suggesting a potential role of Hpn to alleviate the toxicity by sequestering excess intracellular metal ions. Hpn binds five nickel ions per monomer at pH 7.4,[7] and under physiologically relevant conditions, it is found in an equilibrium of multimeric forms with 20-mers (ca. 136 kDa) being the predominant species.

Several bacterial His-rich proteins/motifs are known and have been suggested to be involved in Ni^{2+} metabolism. The Ni^{2+} dissociation constants (K_d) of these proteins have been reported to be 1.3, 2.3, 1.0, 0.6, and 7.1 μM for HypA,[8] HypB,[9] UreE,[10] HspA,[11] and Hpn,[7] respectively. Thus, it is thermodynamically possible for urease chaperones UreE and HypA[12] to remove Ni^{2+} from Hpn under nickel-deficient conditions,[13] if the in vitro K_d values can be extrapolated to in vivo conditions.

To further characterize the metal binding properties of Hpn, stoichiometries and affinities for essential metals Cu^{2+} and Zn^{2+} and a therapeutic metal Bi^{3+} (a clinically used drug for *H. pylori* infection)[14] were determined by equilibrium dialysis together with ICP−MS. The protein, which was expressed and purified as described previously,[7] was found to bind 8.46 (±0.07) Cu^{2+} per monomer with a K_d of 2.16 (±0.04) μM, Figure 1. Hpn also binds to the same amount of Zn^{2+} (5.11 (±0.21) per monomer) as Ni^{2+}, but its Zn^{2+} affinity is 9-fold and 2.7-fold weaker than that for Cu^{2+} and Ni^{2+} (K_d, 7.1 μM), respectively, with a K_d of 19.28 (±1.69) μM. This suggests that Hpn may be involved in the homeostasis of Cu^{2+} and Zn^{2+}, as well as Ni^{2+},[7] although this may not be the case for copper, whose homeostasis involves Cu^{+} in the reducing cellular environment. The order of affinities for these divalent metal ions is Cu^{2+} (2.16 μM) > Ni^{2+} (7.10 μM) > Zn^{2+} (19.28 μM). Surprisingly, Hpn was found to bind only 3.81 (±0.23) Bi^{3+} per Hpn monomer with a K_d of 11.11 (±1.38) μM, when bismuth nitrate was used. The number of Bi^{3+} bound to Hpn dropped to ~1 when bismuth citrate was used, indicating a competition between citrate and the protein. Binding of Bi^{3+} to Hpn suggests a role for Hpn in *H. pylori* response to the bismuth therapy, which has widely been recommended for the treatment of *H. pylori* infection.[14] The range of metal-binding stoichiometries for Hpn, Cu^{2+} > Ni^{2+} ≈ Zn^{2+} > Bi^{3+}, is probably due to different coordination preference of the metal ions. It seems unlikely to be due to the different predominant oligomerization of the protein by the metal ions, since Hpn saturated with Cu^{2+}, Zn^{2+}, or Bi^{3+} had similar gel filtration profile to that of Ni^{2+} form.[7]

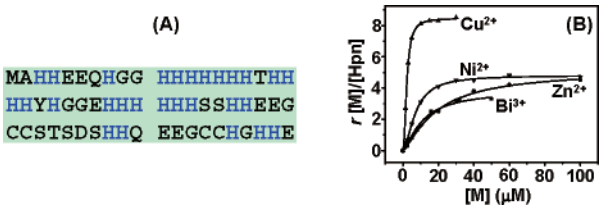

Figure 1. Amino acid sequence of Hpn with histidine highlighted (A) and binding of Cu^{2+}, Ni^{2+}, Zn^{2+}, and Bi^{3+} to Hpn (B). Hpn was dialyzed overnight against 20 mM HEPES, pH 7.4, and 100 mM NaCl in the presence of appropriate amounts of $CuSO_4$, $ZnSO_4$, $NiSO_4$, or $Bi(NO_3)_3$.

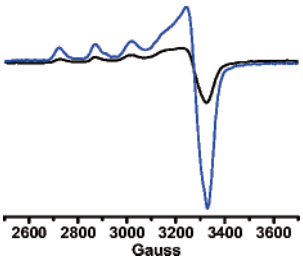

Figure 2. Frozen solution (77 K) EPR spectra of Hpn (0.1 mM) with 1 and 5 mol equiv of Cu^{2+} (black and blue lines) in 100 mM Tris at pH 8.1.

To understand the metal coordination to the protein, a Cu^{2+} titration of Hpn was monitored by EPR spectroscopy (Figure 2). Identical Cu^{2+} EPR spectra were observed for the metal−protein complex at all stoichiometries at pH 8.1, indicating similar ligation and coordination for all Cu^{2+} bound to Hpn. The $g_{||}$ and $g_{\perp}$ values are 2.285 and 2.060, respectively, while the $A_{||}$ is 1.56×10^{-4} cm^{-1}. Comparing these $g_{||}$ and $A_{||}$ values with those from other Cu^{2+} containing proteins and model complexes,[15] it appears that the tetragonal Cu^{2+} ions have a 4N (4 histidines) or 3N1O/S (three histidines and one oxygen/sulfur donor ligand) ligand environment. Surprisingly, EPR spectral evidence for the expected square planar Cu^{2+} coordination at the N-terminal AHH is not observed.[16] Since Cu^{2+} and Ni^{2+} form stable complexes with the N-terminal XXH motif in peptides and proteins, such as albumin,[17] its absence in Hpn suggests that the abundant histidine residues effectively out-compete or disrupt Cu^{2+} and presumably Ni^{2+} coordination at this site.

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10.1021/ja062589t CCC: $33.50

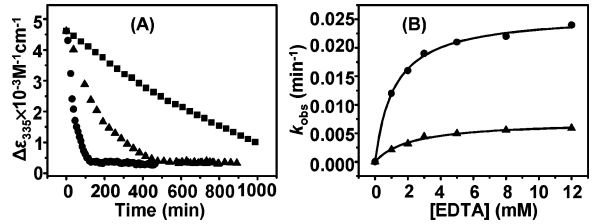

Figure 3. (A) Kinetics of Ni^{2+} release from nickel-saturated Hpn (0.1 mM) by EDTA (12 mM) at pH 6.8 (●), 7.4 (▲), and 8.0 (■) at 298 K. (B) The apparent rate of nickel release versus concentrations of EDTA at pH 6.8 (●) and pH 7.4 (▲) and the best nonlinear fit of the data.

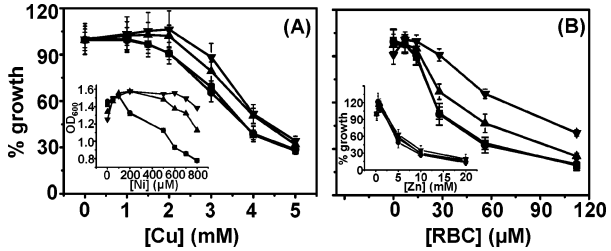

Figure 4. Altered *E. coli* sensitivity to Cu^{2+} (A) and Bi^{3+} (B) in *E. coli* in the presence and absence of Hpn expression at 310 K. Symbol code: ▼, *+hpn*, with IPTG induction (0.5 mM); ▲, *+hpn*, no IPTG induction; ●, *−hpn*, with IPTG induction (0.5 mM); ■, *−hpn*, no IPTG induction.

A competition binding assay between Hpn and EDTA for Ni^{2+} was examined by observing the spectral changes at 298 K (Figure 3A). Upon addition of 120-fold EDTA to the Ni^{2+}-saturated Hpn solution at pH 8.0, the absorption at 335 nm decreased exponentially with a half-time of ca. 600 min, indicative of a gradual release of Ni^{2+} from Hpn. In contrast, the absorption at 335 nm decreased faster at pH 7.4 ($t_{1/2} \approx 110$ min), and even faster at pH 6.8 ($t_{1/2} \approx 41$ min) under identical conditions, suggesting that the protonation state of the histidine residues governs not only affinity but also Ni^{2+} exchange kinetics. As reported previously,[7] the peak was restored to original levels upon adding excess nickel ions, confirming that nickel binding to Hpn is reversible.

The apparent rate constant, k_{obs}, was found to be dependent on the EDTA concentration at both pH 6.8 and 7.4, and to exhibit saturation at high EDTA concentrations (Figure 3B). This could be explained by a two-step process consisting of a rapidly established equilibrium (formation of Hpn−Ni···EDTA, *K*) followed by a rate-determining step (dissociation of Hpn−Ni···EDTA to Ni−EDTA and apo−Hpn, k_2) with a rate law of

$$k_{obs} = k_2[\text{EDTA}]/\{[\text{EDTA}] + (1/K)\}$$

Rate constants (k_2) of 2.59×10^{-2} and 7.09×10^{-3} min^{-1} and *K* values of 8.50×10^2 and 4.53×10^2 M^{-1} were determined at pH 6.8 and 7.4, respectively, suggesting that lower pH favors both formation of the Hpn−Ni···EDTA intermediate and its decomposition to the Ni-exchanged products.

Previously, we have shown that introduction of the *hpn* gene into *E. coli* could provide protection from increased concentrations of Ni^{2+}.[7] To further investigate the potential metal specificity of Hpn in vivo, we performed similar studies with Cu^{2+} (as $CuSO_4$), Zn^{2+} (as $ZnSO_4$), and Bi^{3+} (as RBC) (Figure 4). The effects of metals on the growth of the *E. coli* BL21(DE3) cells were examined by measuring the OD_{600} values. It was found that cells with the *hpn* gene grew slightly better upon addition of IPTG than those without, whereas those cells without the *hpn* grew much worse than those with *hpn* at $[Bi^{3+}] > 30$ μM. Addition of $NiSO_4$ to the medium showed more pronounced differences between the strains with and without the *hpn* gene (Figure 4A, inset).[7] Western blot experiments showed that more Hpn is expressed in the presence of Ni^{2+} (data not shown). In contrast, cells showed comparable growth at $[Cu^{2+}] > 2$ mM and at $[Zn^{2+}] > 5$ mM (Figure 4A,B, inset). Therefore the in vivo protection provided by Hpn follows the order of $Ni^{2+} > Bi^{3+} > Cu^{2+} \approx Zn^{2+}$, despite its higher affinity for Cu^{2+} in vitro. The experiments were also carried out anaerobicly; however, the cell growth was too slow, preventing further investigation under this condition. The comparison between in vitro results (metal affinities) and putative in vivo function (sequestering toxic levels of metal ions) suggests two cautionary observations. First, in vivo interactions of Hpn with metal ions reflect not only metal affinity, but also competition with other cellular proteins. Since copper and zinc are also essential for the host, proteins involved in their homeostasis are expected to be present at high concentrations, which will limit their in vivo concentrations. Second, free intracellular copper[18] likely exists as Cu^{+}, which further compromises the relevance between in vitro Cu^{2+} affinity and in vivo function. Finally, it is useful to note structural and functional similarity between Hpn and metallothionein;[19] both are small proteins rich in one type of metal-binding residue with relatively high metal binding stoichiometries and affinities.

In summary, the histidine-rich protein Hpn represents a novel class of metal binding proteins with the relative binding affinities: $Cu^{2+} > Ni^{2+} > Bi^{3+} \approx Zn^{2+}$. However, its in vivo selectivity, as indicated by protection against toxic levels of metals, is $Ni^{2+} > Bi^{3+} > Cu^{2+} \approx Zn^{2+}$, suggesting a primary role in Ni^{2+} storage and homeostasis for *H. pylori*. Since many histidine-rich motifs and proteins are found in microorganisms, this study provides a new insight into their metal binding properties and in vivo functions in metal homeostasis.

Acknowledgment. This work was supported by the RGC, the RFCID, and the CA and AoE Scheme of the UGC.

Supporting Information Available: Experimental data and complete ref 5a. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA062589T