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The *Helicobacter pylori* HypA•UreE₂ complex contains a novel high-affinity Ni(II) binding site

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Abstract

Helicobacter pylori is a human pathogen that colonizes the stomach, is the major cause of ulcers, and has been associated with stomach cancers. In order to survive in the acidic environment of the stomach, *H. pylori* uses urease, a nickel-dependent enzyme, to produce ammonia for maintenance of cellular pH. The bacteria produce apo-urease in large quantities and activate it by incorporating nickel under acid shock conditions. Urease nickel incorporation requires the urease-specific metallochaperone UreE and the (UreFGH)₂ maturation complex. In addition, the *H. pylori* nickel urease maturation pathway recruits accessory proteins from the [NiFe] hydrogenase maturation pathway, namely HypA and HypB. HypA and UreE dimers (UreE₂) are known to form a protein complex, the role of which in urease maturation is largely unknown. Herein, we examine the nickel binding properties and protein-protein interactions of HypA and UreE₂ using ITC and fluorometric methods under neutral and acidic pH conditions for insights into the roles played by HypA in urease maturation. The results reveal that HypA and UreE₂ form a stable complex with μ M affinity that protects UreE from hydrolytic degradation. The HypA•UreE₂ complex contains a unique high-affinity (nM) Ni²⁺ binding site that is maintained under conditions designed to mimic acid shock (pH 6.3). The data are interpreted in terms of a proposed mechanism wherein HypA and UreE₂ act as co-metallochaperones that target Ni²⁺ delivery to apo-urease with high fidelity.

Graphical Abstract

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Author Contributions

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ASSOCIATED CONTENT

Supporting Information with Materials/Experimental Details and supporting figures.

Keywords

Helicobacter pylori; HypA; UreE; metallochaperones; urease maturation; nickel insertion; protein-protein interactions; Ni-protein interactions

INTRODUCTION

Helicobacter pylori is a successful human pathogen that colonizes the stomach and causes chronic gastritis, ulcers, and cancers.^{1, 2} Despite thriving in the niche environment of the acidic stomach, *H. pylori* is a neutrophilic bacterium. One of the adaptations that allow for successful colonization in the acidic stomach is the ability to rapidly increase urease activity upon acid shock.³ Urease catalyzes the hydrolysis of urea to ammonia and carbamate (spontaneously hydrolyzing to carbon dioxide and ammonia),⁴ which neutralizes the intracellular pH of the bacterium in acidic environments.⁵ Acid readiness is ensured by a plentiful supply of apo-urease protein, which accounts for 7 - 10% of the total nascent proteins synthesized by *H. pylori* at both neutral and acidic pH conditions.⁶ Activation of urease involves the assembly of the di-nickel active sites, which requires the accessory proteins UreEFGH as well as hydrogenase-associated accessory proteins HypA and HypB in *H. pylori*.^{7, 8} The function of the accessory proteins in urease maturation has been extensively studied and reviewed for *H. pylori* and other organisms.^{7, 8}

Of these accessory proteins, UreE has been identified as a Ni-metallochaperone responsible for acquiring and specifically delivering Ni²⁺ for urease activation.⁷ *H. pylori* UreE is a homodimer (UreE₂), where a six-coordinate Ni site is formed at the dimer interface using a labile C-terminal motif.⁹ The interfacial UreE₂ Ni-binding site has an apparent dissociation constant (K_d) of $0.15 \pm 0.01 \mu\text{M}$, as measured by isothermal titration calorimetry (ITC).¹⁰ UreE homologues from other organisms have evolved Ni-sequestration ability employing a His-rich tail, which is lacking in *H. pylori* UreE,¹¹ resulting in a lower Ni-binding capacity of one Ni²⁺ per dimer⁹⁻¹² compared with up to six Ni²⁺ per dimer in *K. aerogenes* UreE₂.^{7, 13, 14} Appending His-rich tails derived from homologues to the *H. pylori* UreE protein sequence increases the Ni-binding capacity of the protein variants and the urease activities of corresponding *H. pylori* isostrains.¹¹

Urease activity in *H. pylori* also requires another known Ni-metallochaperone, HypA,¹⁵ which is typically associated with the maturation of [NiFe] hydrogenases.¹⁶ Interruption of the *hypA* gene^{15, 17, 18} or diminishing its Ni-binding ability by site-directed mutagenesis^{18, 19} have been shown to abolish urease activity and acid survival of *H. pylori* cells. WT-like urease activity in *hypA*-interruption strains of *H. pylori* can be restored by Ni-supplementation in the growth media,¹⁵ but cannot rescue *ureE*-interruption strains.²⁰ These phenotypes suggest that while both HypA and UreE₂ are involved in delivery of Ni²⁺ to *H. pylori* apo-urease, Ni²⁺ insertion specifically requires UreE₂, potentially due to protein-protein interactions with downstream urease accessory proteins in a maturation complex,²¹ such as UreG,^{10, 22-26} and as observed in other bacterial strains.⁷

Purified HypA protein has been shown to interact with UreE₂ forming a stable complex that can outcompete complex formation with UreG,²⁷ an accessory protein in the urease maturation cascade that is characterized as a GTPase.^{21, 28-30} In contrast, alternate studies using N-terminally modified HypA (where cleavage of an affinity tag left a Gly-Ser overhang at the N-terminal Ni-binding motif; henceforth referred to as GS-HypA) found that the GS-HypA•UreE₂ complex dissociates in favor of a UreG₂•UreE₂ complex in the presence of Mg and GTP.²² The C-terminal motif of *H. pylori* UreE (residues 158 - 170) is essential for interaction with HypA.³¹ The C-terminal motif is disordered in crystal structures.^{9, 12} Truncation of the C-terminal 10 and 12 residues in the UreE peptide have been detected by mass spectrometry of purified proteins,¹² which was reported to be remediated by addition of protease inhibitors during purification.¹² Truncation was also reported to affect the number of sites detected in HypA binding to UreE₂ in ITC.¹⁸

H. pylori HypA has been extensively characterized and has been found to be a monomeric protein that contains a structural Zn(Cys)₄ site, as well as a Ni-binding site with low μM affinity ($K_d \sim 1 \mu\text{M}$).^{18, 32-34} Ni-binding to HypA is approximately one order of magnitude weaker than that of UreE₂, and with both proteins serving the same function of Ni²⁺ acquisition and delivery, the requirement of HypA in *H. pylori* urease maturation seems redundant and confounding. The disparity in the Ni-binding affinities between HypA (Figure 1, Step 1A^o) and UreE₂ (Figure 1, Step 1A*) led to a model in the literature²² that suggests that HypA first acquires Ni²⁺, possibly by direct interaction with the Ni importer, (Figure 1, Step 1A^o), and then transfers it to UreE₂ (Figure 1, Step 1B^o) through protein-protein interactions.^{20, 22} Subsequently, HypA dissociates (Figure 1, Step 1B*) from the complex leaving Ni-UreE₂ to interact with UreG (Figure 1, Step 2) in delivering Ni²⁺ to apo-urease.^{22, 23} In an effort to clarify the model, we measured the binding of the equilibria in Step 1 at pH 7.2 and 6.3, including the interaction of apo,Zn-HypA with apo-UreE₂ (Figure 1, Step 1B[^]), and Ni interaction with the apo,Zn-HypA•UreE₂ protein complex (Figure 1, Step 1A[^]).

Early *in vitro* and *in vivo* attempts to show Ni-transfer between HypA and UreE₂ yielded ambiguous results.²⁰ Ni-transfer from GS-HypA to UreE₂ has been demonstrated using a Ni-specific cross-linking dye after SDS-PAGE separation of the two proteins.³¹ However, the N-terminal modification in the GS-HypA variant has significantly weakened the Ni-binding³¹ compared to wild type.^{18, 33} This was demonstrated in other N-terminally modified HypA variants, such as H2A, which completely abrogated Ni-binding.^{19, 20}

Another variant, M1_H2insL (hereafter known as L2*-HypA) exhibits 60-fold weaker Ni-binding compared to wild type. Additionally, separation of the tightly bound Ni,Zn-GS-HypA•UreE₂ complex requires denaturing conditions³¹ or additional cofactors, such as Mg, GTP, and UreG₂.²²

As a mechanism accounting for the HypA-requirement in *H. pylori* urease maturation, the HypA Ni²⁺ acquisition and handoff to UreE₂ is unsatisfactory in two respects: First, the *in vitro* studies of HypA•UreE₂ interaction reported thus far have been performed under neutral pH conditions, which may miss key aspects of the mechanism of Ni-delivery for urease maturation that are critical for the survival of *H. pylori* under acid shock (with internal pH ~ 6.3).³⁵⁻³⁷ Second, the studies performed thus far at neutral pH indicate that the HypA•UreE₂ complex is stable with or without Ni²⁺,^{27, 31} which renders spontaneous HypA dissociation from the complex unlikely. Although UreE₂ binds Ni²⁺ more tightly than does HypA,^{10, 18, 33} the protein dissociation predicted in the current model is hampered by their strong protein-protein interactions.

The goal of the studies presented here is to further elucidate the role(s) of HypA in Ni²⁺ delivery to the urease maturation pathway in *H. pylori* by probing *in vitro* pH effects that mimic acid shock (*i.e.*, at pH 6.3). Although many of these binding events were previously characterized under neutral conditions,^{10, 18, 27, 31, 33} they were re-characterized in this study employing a single buffer system to facilitate comprehensive comparisons between pH and proteins. Buffer NTP (20 mM HEPES, 100 mM NaCl, 100 mM KCl, 5 mM MgCl₂, 1 mM TCEP, at pH 7.2 or 6.3) was chosen as the unifying buffer system because future studies involving these proteins will likely involve the GTPases, HypB and UreG, the structures and activities of which may depend on reducing agents, K⁺, and Mg²⁺.^{21, 23, 38} We found that the HypA•UreE₂ complex contains a novel high-affinity Ni-binding site.

MATERIALS/EXPERIMENTAL DETAILS

Detailed descriptions of materials and methods can be found in the supporting information.

RESULTS

Low pH does not change the relative nickel binding affinities of HypA and UreE₂ metallochaperones.

Prior studies have established that Ni binds to *H. pylori* UreE₂ approximately one order of magnitude tighter than HypA at neutral pH,^{10, 31, 33} but Ni²⁺ binding to UreE₂ has not been studied under conditions resembling acid shock. It is possible that under acid shock conditions Ni²⁺ binding to HypA (Figure 1, Step 1A°) is favored over UreE₂ (Figure 1, Step 1A*), and this would provide a requirement for HypA in urease maturation under acid shock conditions.

Using identical buffers, Ni²⁺ binding to HypA and UreE₂ under neutral (pH 7.2) or acid shock (pH 6.3) conditions were compared. NiCl₂ was dissolved in buffers at pH 7.2 or 6.3, and then titrated into either apo-UreE₂ or apo,Zn-HypA in the same buffer, monitoring the heat of binding using ITC. A single isotherm was observed for Ni²⁺ binding to either apo-

UreE₂ or apo,Zn-HypA, and the data are best fit by OneSite models in both cases, as summarized in Table 1, with example data and fits shown in Figure 2. The modest change in pH conditions did not significantly alter the Ni²⁺ binding affinity of either UreE₂ or HypA, and UreE₂ bound Ni²⁺ at least one order of magnitude tighter than HypA under both pH conditions.

The K_d value and number of sites (N) determined for Ni binding to apo,Zn-HypA were successfully reproduced using the new buffer system at pH 7.2, and in good agreement with prior reports,^{18, 33} our results at pH 6.3 disagree with a previous study that found 15-fold weaker binding as compared to neutral pH and only one Ni per dimer ($N \sim 0.5$).³³ HypA was previously thought to be a dimer in solution,³³ but was later shown to be a monomer by size exclusion chromatography in tandem with multi-angle light-scattering (SEC-MALS).¹⁸ This discrepancy might be attributed to buffer system differences, where the presence of reducing agents in the buffer used in these studies prevented cysteine oxidation, etc., and therefore better maintained the native Zn-bound state of HypA.

Additionally, we observed that Ni binding to apo,Zn-HypA is exothermic at pH 7.2 and endothermic pH 6.3. This enthalpy change may indicate that at least one of the Ni-binding ligands has a pK_a below 7.2 as previously suggested,³³ or that additional protons in the Ni-binding pocket of the protein at lower pH must be displaced during Ni binding. (An endothermic binding event was similarly observed in Ni binding to the HypA•UreE₂ complex at pH 6.3, but not at pH 7.2, *vide infra*).

The ITC data for Ni-binding to UreE₂ was previously characterized at pH 7.¹⁰ We repeated the titrations in our buffer systems in order to compare Ni-binding to different proteins and protein complexes. Our K_d at pH 7.2 is approximately 2-fold tighter than the previously reported data,¹⁰ which may be due to differences in our buffer systems. Moreover, our ITC data also reveal substoichiometric Ni-binding for UreE₂ at both pH values (Table 1), which is in contrast to the previously reported $N = 1$ at pH 7.0.¹⁰ This can be attributed to hydrolytic degradation of UreE₂, which was confirmed by SDS-PAGE after ITC experiments,¹⁸ and ESI-mass spectrometry (*vide infra*). As previously reported, the C-terminal motif that is close to the Ni-binding site in *H. pylori* UreE₂ degrades slowly over time.¹⁸ Thus, the N values determined in our fits are consistent with one Ni-site per UreE₂ dimer where a portion of the sample has degraded. The optimal 1:1 stoichiometry is consistent with previously reported crystal structures^{9, 12} and Ni K-edge X-ray absorption spectroscopy data.⁹

Apo,Zn-HypA•UreE₂ interactions are not altered by acid shock conditions.

Prior studies of the N-terminally modified GS-HypA and UreE₂ mixture by SEC and Static Light Scattering performed at neutral pH indicated the formation of a stable GS-HypA•UreE₂ complex with a mass of 51 kDa.³¹ If the HypA•UreE₂ interaction (Figure 1, Steps 1B[^] and 1B[°]) were enhanced by acidic pH, recruiting HypA to the urease maturation pathway through protein-protein interactions under acidic shock conditions might be involved in urease maturation.

To assess the pH effects on the interaction between apo,Zn-HypA or Ni,Zn-HypA with UreE₂, SEC-MALS and ITC titrations were performed under neutral (pH 7.2) and acid shock (pH 6.3) conditions, and reveal a stable complex at both pH values. SEC-MALS was used to determine the masses of peaks resolved from a mixture containing a three-fold excess of apo,Zn-HypA to apo-UreE₂ at both pH 7.2 and 6.3. Two peaks were clearly resolved for the mixture under both pH conditions with elution volumes of 7.2 - 7.3 and 8.5 - 8.7 mL (Supporting Information, Figure S2A). The peak at 8.5 - 8.7 mL overlaps with the peak for isolated apo,Zn-HypA, has a molecular mass of 13 kDa, and is therefore attributable to the excess apo,Zn-HypA protein in the 3:1 mixture. The peak at 7.2 - 7.3 mL has a molecular mass of 51 kDa, matching the mass of the previously characterized GS-HypA•UreE₂ complex,³¹ and is distinct from either apo,Zn-HypA or apo-UreE₂ alone (eluting at 7.6 - 7.8 mL with a mass of 38 kDa). This peak is therefore assigned to a HypA•UreE₂ complex.

The formation of the apo,Zn-HypA•UreE₂ complex was also assessed by titration of apo-UreE₂ with apo,Zn-HypA measured by ITC. Single isotherm binding curves were detected at both pH 7.2 and 6.3 (Figure 3, left) and best fitted by OneSite models with apparent dissociation constants (K_d) in the μ M range (Table 2). These K_d values reproduced previous ITC studies of apo,Zn-HypA binding to UreE₂,¹⁸ and corroborated with the GS-HypA binding to apo-UreE₂ performed at neutral pH,³¹ as well as a μ M binding event detected by bio-layer interferometry (BLI).²⁷ The change in pH from 7.2 to 6.3 did not affect the overall binding of apo,Zn-HypA to apo-UreE₂, suggesting that acid shock conditions do not alter the interaction between these proteins in the absence of Ni.

Fluorescence intensity change was also used to examine apo,Zn-HypA•UreE₂ binding. N-hydroxysuccinimide (NHS)-ester chemistry was used to label primary amines in purified recombinant apo-UreE₂ with a fluorophore (NHS640-UreE₂). Primary amines from lysine side-chains are distributed throughout the UreE₂ protein, but none are close to the Ni-binding site (PDB ID: 3TJ8⁹, and 3NY0¹²). The ratio of fluorophore-to-UreE₂ was between 0.4 - 1.2, which was quantitated by UV-vis (see Materials/Experimental Details in the Supporting Information). Ni²⁺ titration into NHS640-UreE₂ (Figure S3A) confirms that Ni-binding on fluorescently-labeled UreE₂ is not altered.

A constant amount NHS640-UreE₂ was mixed with increasing concentrations of apo,Zn-HypA, and the fluorescence of each mixture was then measured using a NanoTemper NT. 115. Fluorescence intensity increases from apo,Zn-HypA titration into apo-NHS640-UreE₂ were fitted to a modified mass action function assuming a single binding site to obtain K_d with μ M values at pH 7.2 and 6.3 (Figure 4 left panels and Table 2). The results obtained corroborate the ITC results and support a single observable binding event between apo,Zn-HypA and apo-UreE₂.

The apo,Zn-HypA•UreE₂ complex contains a novel high-affinity Ni binding site.

ITC was used to measure the Ni²⁺ binding to the apo,Zn-HypA•UreE₂ complex under neutral (pH 7.2) and acid shock (pH 6.3) conditions to assess whether Ni-binding is enhanced in the protein complex. The formation of the HypA•UreE₂ complex under the conditions used in these titrations was confirmed by protein-protein titrations and SEC-

MALS (see Table 2, Figure 3 and Figure 4 left panels, and Supporting Information, Figure S2). Two distinct isotherms were observed in the titration of the apo,Zn-HypA•UreE₂ complex with Ni²⁺ at both pH 7.2 and 6.3, and were best fitted to models that featured two independent sites (Figure 5 right panels, Table 1). Fits for a single binding site model produced statistically and visually poorer fits (Supporting Information, Figure S3B). At both acidic and neutral pH, the weaker affinity binding event (K_{d2} in Figure 5, right panels) featured μM K_d values that are similar to those for Ni²⁺ binding to HypA (Table 1). The tighter binding events (K_{d1} in Figure 5, right panels) featured low- to sub-nM K_d values. At neutral pH, this sub-nM binding event is almost three orders of magnitude tighter than the measured K_d for Ni²⁺ binding to UreE₂ (Table 1). At pH 6.3, the nM binding event is one-order of magnitude tighter than the measured K_d for Ni²⁺ binding to UreE₂ (Table 1). Thus, the interaction of Ni²⁺ with the apo,Zn-HypA•UreE₂ complex (Figure 1, Step 1A[^]) is distinct from the combination of Ni-binding to each protein individually (Figure 1, Step 1A[°] and 1A^{*}). Due to the complexity of the system, it is unclear whether both isotherms observed in the titration of apo,Zn-HypA•UreE₂ with Ni can be attributed to Ni-binding. Alternatively, one of the isotherms may be the result of Ni-dependent protein conformational change, which was previously proposed in BLI studies.²⁷

Although HypA has only μM affinity towards Ni²⁺ (Table 1), it plays a crucial role in the high-affinity Ni-binding event observed in the apo,Zn-HypA•UreE₂ complex. A previously characterized HypA variant, L2*-HypA, which alters the ability of the N-terminal amine to serve as a Ni ligand, was shown to bind Ni²⁺ at a lower affinity than WT-HypA (Table 1) without affecting the binding affinity of the HypA protein to UreE₂ (Table 2).¹⁸ L2*-HypA closely resembles the GS-HypA variant in terms of Ni-binding affinity ($K_d \sim 14 \mu\text{M}$),³¹ Ni-site structure, and magnetic properties.^{18, 34} Titration of the complex formed between apo,Zn-L2*-HypA and UreE₂, apo,Zn-L2*-HypA•UreE₂, with Ni²⁺ was measured by ITC (Figure 5, left panels). In contrast to titration of wild type apo,Zn-HypA•UreE₂ (Figure 5, right panels), only a single isotherm with weaker binding was observed for apo,Zn-L2*-HypA•UreE₂ at either pH, with K_d values that are similar to those obtained from Ni²⁺ binding to apo-UreE₂ (Table 1). The higher affinity binding event (K_{d1} in Figure 5, right panels) observed in the Ni²⁺ titration into wild type apo,Zn-HypA•UreE₂ is absent. Thus, the loss of this higher affinity binding event upon the disruption of the native HypA Ni-binding site shows that the formation of the novel high-affinity site involves the N-terminal amine of HypA, a known Ni ligand in *H. pylori* HypA.¹⁸

ITC was also used to monitor the interaction between HypA and UreE₂ when the Ni complex of HypA, Ni,Zn-HypA, was used as the titrant under both neutral (pH 7.2) and acid shock (pH 6.3) conditions (Figure 1, Step 1B[°]). The results are consistent with a series of complex interactions that are reflected in the observation of at least two distinct isotherms under both pH conditions. TwoSites models were used to fit the titrations at both pH values and reveal two binding events (Figure 3, right). The tighter binding events (K_{d1} in Figure 3, right panels) were best fitted with nM K_d values at both neutral and acidic pH values and are two orders of magnitude tighter than the binding between the apo-proteins observed in the absence of Ni²⁺ (Table 2). These K_d values are of the same order of magnitude as the high-affinity binding events observed in the titration of the apo,Zn-HypA•UreE₂ complex with Ni²⁺, although it is unclear whether the same binding events account for these isotherms.

The weaker affinity events (K_d in Figure 3, right panels) were best fitted with sub- μM K_d values (Table 2), which are similar to Ni^{2+} binding to apo-UreE₂ (Table 1). The tighter binding events are only observed in the presence of both Ni and the HypA•UreE₂ complex (*i.e.*, in the titrations of Ni,Zn-HypA into apo-UreE₂; or in the titrations of Ni^{2+} into the apo,Zn-HypA•UreE₂ complexes). This suggests that the tighter protein interactions are due to the formation of the novel Ni-binding site formed in the apo,Zn-HypA•UreE₂ complex that is absent in the individual proteins.

ITC detects all the processes that result in heat change during a titration, including any metal binding/unbinding and changes to protein conformation. To simplify the processes observed in the titration of apo-UreE₂ with Ni,Zn-HypA, the titration was repeated under saturating Ni^{2+} conditions using fluorescence change as a detection method. The fluorescence change of Ni-NHS640-UreE₂ was measured upon titration with unlabeled Ni,Zn-HypA under both neutral (pH 7.2) and acid shock (pH 6.3) conditions in the presence of excess Ni^{2+} (1 μM) in the buffer; Figure 4 and Table 2). In this approach, the signal is derived exclusively from fluorophores bound to the surface of Ni-NHS640-UreE₂ at nM concentration, where the UreE₂ Ni-binding site is saturated. The release or rebinding of Ni^{2+} or rearrangements in the unlabeled HypA are not detectable by this method unless it is directly bound to the surface of the Ni-NHS640-UreE₂. This experimental design allowed for the detected signals to be unambiguously assigned to protein-protein interactions in the presence of Ni.

In Figure 4, the changes in fluorescence intensities are plotted against the concentration of Ni,Zn-HypA and used to generate binding curves. At pH 7.2, the binding of Ni,Zn-HypA to Ni-NHS640-UreE₂ was fitted to a modified mass action function, assuming a single binding site, resulting in a $K_d = 20 \pm 9$ nM. The binding at pH 6.3 was best fitted with Hill's equation, resulting in $K_d = 4.5 \pm 2.6$ nM with a Hill coefficient (H) of 1.4 ± 0.6 , suggesting some small cooperativity in binding. These K_d values are at least two-orders of magnitude tighter than the apo-protein interactions measured by fluorescence at both pH values ($K_d \sim 2$ μM). These observations unambiguously establish that a HypA•UreE₂ complex forms under saturating Ni^{2+} conditions, and that the interactions between Ni,Zn-HypA and Ni-UreE₂ are *enhanced* under both neutral and acid shock conditions relative to the situation without Ni present. Further, the complex is about 10-fold tighter under acid shock conditions. The overall enhanced protein interactions provide a possible mechanism for increased Ni delivery to apo-urease under acid shock conditions and suggest a more involved role for HypA beyond the handoff of Ni^{2+} to UreE₂.

The Ni-NHS640-UreE₂ fluorescence intensities decrease with increasing concentration of Ni,Zn-HypA, which is opposite to the effect observed for the apo,Zn-HypA interaction with apo-NHS640-UreE₂ (Figure 4). The reversal of the fluorescence intensity changes in response to increasing concentrations of HypA indicates that interactions that occur in the Ni-bound proteins are distinct from the binding events in the apo-proteins, and the presence of Ni may be quenching the fluorescence.

To assess the stability of the Ni-HypA•UreE₂ ternary complex, particularly its ability to retain Ni^{2+} ions under dilute conditions, the SEC-MALS experiment described above for the interaction of apo,Zn-HypA and apo-UreE₂ was repeated using Ni,Zn-HypA. A three-fold

excess of Ni,Zn-HypA was mixed with apo-UreE₂ under neutral (pH 7.2) and acidic (pH 6.3) conditions, and then separated and analyzed by SEC-MALS. The results shown in Figure 6 are essentially identical to those obtained using apo,Zn-HypA and apo-UreE₂ mixtures (Supporting Information, Figure S2A), and featured two resolved peaks at similar elution volumes. The first peak, centered at elution volumes of 7.1 - 7.3 mL, corresponds to a molecular mass of 51 kDa at both pH 7.2 and 6.3 and is assigned to the stable complex of Zn-HypA•UreE₂ with or without Ni²⁺ bound. A second smaller peak, eluted at 8.5 - 8.7 mL, overlaps with the peak assigned to HypA. This peak has a molecular mass of 13 kDa at both pH values, and is assigned to the excess HypA, with or without Ni²⁺ bound.

To assess whether metals remain bound to the resolved proteins, each peak was collected and analyzed for metal content using inductively coupled plasma optical emission spectroscopy (ICP-OES). Since properly folded HypA contains a structural Zn-site,^{17, 32, 33, 39} comparing the Ni:Zn ratio of each peak is a convenient way to accurately assess the Ni retention and stoichiometry compared with the amount of HypA in the sample. The 51 kDa HypA•UreE₂ complex contained 0.36 ± 0.06 μ M Ni with a Ni:Zn ratio of 1.1 at pH 7.2; and had a 0.10 ± 0.05 μ M Ni concentration with a Ni:Zn ratio of 1.3 at pH 6.3. These results show that both Ni and Zn are retained in the protein complex and this complex is stable at sub-micromolar concentrations (Figure 1, Path 1B). The peak corresponding to excess Ni,Zn-HypA contained 0.72 ± 0.05 μ M Ni with a Ni:Zn ratio of 0.85 at pH 7.2; and contained 0.38 ± 0.03 μ M Ni with a Ni:Zn ratio of 0.98 at pH 6.3, demonstrating that Ni,Zn-HypA also retains Ni under these conditions at close to 1:1 ratios (Figure 1, Step 1B*). Together these data indicate that the Ni,Zn-HypA•UreE₂ complex is stable at sub-micromolar concentrations, suggesting that this complex is unlikely to spontaneously dissociate.

The stable HypA•UreE₂ complex was also observed with SEC-MALS separation of 1:1 mixtures of Ni,Zn-HypA with UreE₂ at both pH 7.2 and 6.3 (Figure S2B). Although Ni and Zn were detected in this peak, the Ni:Zn ratio was approximately 0.7 at pH 7.2 and 0.5 at pH 6.3. A much smaller second peak with a deconvoluted mass of 35 kDa was also observed, with trace amounts of Ni and Zn were also detected. This small peak may correspond to a mixture of UreE₂ where one or both subunits contain some degree of degradation at the C-terminal domain; and/or a mixture of monomeric UreE₂ in complex with Ni,Zn-HypA.

The Ni,Zn-HypA•UreE₂ complex protects UreE from rapid hydrolytic degradation.

The C-terminal domain of *H. pylori* UreE protein is disordered in crystal structures^{9, 12} and is known to degrade over time, producing two distinct bands on SDS-PAGE (Supporting Information, Figure S4).^{9, 12, 18} LC-MS (Figure S5) analysis of freshly prepared UreE protein identified the full-length protein at 19406.0 Da (theoretical mass = 19407.5 Da) and a major degradation product at 18502.5 Da corresponding to UreE with eight residues missing from the C-terminus (theoretical mass = 18504.5 Da for a UreE 163-170 fragment). Loss of the peak corresponding to the full-length protein and additional degradation products (Figure S5) were detected after five days at room temperature, with masses of 18318.2 and 18231.5 Da, matching the expected masses of UreE peptides missing the C-terminal 10 and 11-residues, (theoretical masses = 18320.21 and 18233.13 Da). A similar *H.*

pylori UreE variant, UreE₁₅₈₋₁₇₀, has been shown to lose the ability to interact with the N-terminally modified GS-HypA,³¹ indicating the C-terminal domain of UreE is critical for the interaction with HypA.

The stability of purified UreE₂ was monitored in the presence and absence of apo,Zn-HypA, with and without of Ni²⁺, at both pH 7.2 and 6.3. At both pH, the C-terminally degraded UreE (UreE_C) peptide fragments increases proportionally to the disappearance of full-length UreE between 0 and 72 hours at 20°C. The rate of degradation is similar in the absence and (Figure 7, lanes 1 - 3) presence of Ni²⁺ (Figure 7, left, lanes 10 - 12) at pH 7.2 and the addition of apo,Zn-HypA (Figure 7, left panel, lanes 4 - 6) also did not significantly alter the degradation rate. However, in the presence of Ni,Zn-HypA (Figure 7 left panel, lanes 7 - 9), the disappearance of full-length UreE peptide was slower, and becoming more apparent after 48 hours (Figure 7 left plot). Complex formation with Ni,Zn-HypA also reduced the degradation of UreE under acid shock conditions (pH 6.3) (Figure 7, right, 7 - 9). No significant degradation of Ni,Zn-HypA was detected during the same time frame, however, up to 50% of the apo,Zn-HypA was degraded along with UreE after 72 hours (Figure 7, lane 6). These observations demonstrate that formation of the Ni,Zn-HypA•UreE₂ complex protected the labile UreE C-terminal domain from hydrolytic degradation and therefore enhances urease maturation by ensuring the availability of full-length UreE.

DISCUSSION

Understanding the mechanism of urease maturation in *H. pylori* is of interest both because the health risks posed by the pervasiveness of the pathogen amongst human hosts, and by the unique adaptations that carefully control urease activity.^{1, 2, 40} One of these adaptations encompasses the conundrum of the HypA-requirement for urease maturation, despite the presence of the urease-specific Ni-metallochaperone, UreE₂.^{15, 41} Using a biophysical approach, the studies presented above identify a novel high-affinity nickel binding site in the HypA•UreE₂ protein complex, address the role of pH in the relevant Ni-protein and protein-protein interactions, and extend current knowledge to include studies that mimic acid shock conditions. The results have important implications for the mechanism of nickel delivery to apo-urease and the role of HypA.

Several hypothetical mechanisms might account for why HypA is needed to achieve full urease activation in *H. pylori*.^{11, 15, 17-20, 23, 31} As the requirement for higher urease activity is most acute under acidic shock, the demand for Ni-insertion into urease would also increase at low pH. Changes in the Ni-binding affinities of the isolated proteins at low pH (Figure 1, Step 1A° and 1A*) were ruled out by titrations conducted at both pH 7.2 and 6.3, an estimate of the internal pH of *H. pylori* under neutral or acid shock conditions (Table 1).³⁵⁻³⁷

Under neutral pH conditions, *H. pylori* HypA and UreE₂ are known to form a stable complex as apo-proteins^{18, 27} and in the presence of metals,^{22, 27} raising the possibility that nickel availability for urease maturation is significantly improved in the Ni,Zn-HypA•UreE₂ complex compared to UreE₂ alone. Improvements in Ni-binding could occur via tighter Ni interaction with the preformed HypA•UreE₂ complex (Figure 1, Step 1A^*) compared to

either protein individually or by enhancing protein-protein complex formation in the presence of Ni^{2+} (Figure 1, Step 1B^o), particularly at low pH. The results of our studies establish that Ni^{2+} binding to the apo,Zn-HypA•UreE₂ complex (Figure 1, Step 1A[^]) is significantly tighter relative to UreE₂ alone at neutral pH and is slightly tighter at pH 6.3 (Table 1).

Enhancing the HypA and UreE₂ protein-protein interactions (Figure 1, Step 1B[^] and 1B^o) under acid shock conditions could also improve Ni delivery to the urease maturation pathway. This was addressed by examining the interaction of apo,Zn-HypA and Ni,Zn-HypA with UreE₂ as a function of pH. The results establish that a single binding event with $K_d \sim 1 - 2 \mu\text{M}$ accounts for the formation of the apo,Zn-HypA•UreE₂ complex (Table 2), which is stable and has the same protein stoichiometry at both pH values (Supporting Information, Figure S2). However, use of Ni,Zn-HypA in the ITC titrations reveals a more complicated binding process that is characterized by at least two binding events, both of which are pH-independent. A high-affinity event ($K_d \sim 0.6 \text{ nM}$) that is not observed in the titration using apo,Zn-HypA, and a lower affinity binding event (with sub- μM K_d) that is similar to the binding of apo,Zn-HypA to apo-UreE₂ (Table 2). Thus confirming the presence of a high-affinity site only in the protein complex. Further studies using fluorescently-labeled NHS640-UreE₂ in the presence of Ni^{2+} shows that high-affinity binding to Ni,Zn-HypA was persistent, where the binding at pH 6.3 ($K_d \sim 4.5 \text{ nM}$) was approximately 4.5-fold improved over pH 7.2 ($K_d \sim 20 \text{ nM}$). Therefore, enhancing the interaction between Ni,Zn-HypA and UreE₂ (Figure 1, Step 1B^o) is a possible mechanism for increasing Ni delivery to the urease maturation pathway under acid shock conditions.

Prior studies of the HypA•UreE₂ interaction at neutral pH with biolayer interferometry (BLI) and surface plasmon resonance (SPR) demonstrated the presence of two binding events (with reported dissociation constants of 5.4 nM and 1.1 μM), which were attributed to initial binding at higher affinity followed by a conformational change at lower affinity.²⁷ The K_d values reported above (Table 2) generally agree with the lower-affinity binding event observed in prior SPR and BLI studies,²⁷ but the previously reported high-affinity site was not detected using the apo-proteins. Addition of Ni^{2+} or Zn^{2+} ions to the binding studies did not affect the binding of HypA and UreE in the BLI and SPR studies,²⁷ but use of Ni,Zn-HypA in the titrations presented above resulted in the observation of a high-affinity binding event by ITC with nM affinity (similar to the high-affinity binding event observed by BLI and SPR). This high-affinity binding event is clearly associated with the presence of Ni^{2+} by the studies presented here. Both the BLI and SPR experiments feature tethering of HypA to a surface, which given its small size (13 kDa) and complexity (binding Zn, Ni, and protein partners), could alter its interaction with UreE₂.

Interactions between HypA and UreE₂ were also studied using the N-terminally modified GS-HypA.³¹ In contrast to the current study, ITC measurements of GS-HypA interaction with UreE₂ produced single isotherms ($K_d \sim 1 \mu\text{M}$), regardless of metal loading of GS-HypA (apo, Zn-bound, or Ni,Zn-bound) or the metal loading of UreE₂ (apo, Zn-bound, or Ni-bound).³¹ The high-affinity binding events ($K_d \sim 0.6 \text{ nM}$) that were observed in the current studies between (Ni,Zn-HypA and apo-UreE₂) and in previous BLI and SPR studies (between tethered-HypA and UreE₂ in the presence and absence of metals)²⁷ were absent in

the GS-HypA studies.³¹ These observations point to a loss of function in the N-terminally modified GS-HypA in Ni-binding, which also interferes with the Ni-site formation in the HypA•UreE₂ complex. Like the GS-HypA variant, the L2*-HypA variant contain similar N-terminal disruptions of the Ni-binding site. The absence of a high-affinity binding event was confirmed when Ni was titrated into the apo,Zn-L2*-HypA•UreE₂ complex (Figure 5).

In our ITC studies of Ni,Zn-HypA binding to apo-UreE₂, both isotherms observed were distinct from those resulting from apo,Zn-HypA interaction with UreE₂ (Figure 3) and therefore cannot be attributed to the same interactions. The isotherms observed for Ni,Zn-HypA titrations of apo-UreE₂ could be the result of combinations of multiple events with distinct heat changes. Some possible events include Ni transfer to UreE₂ from HypA, and/or the dissociation of HypA from Ni-UreE₂ after Ni-transfer, and/or Ni-enhanced HypA•UreE₂ interaction (thermodynamically distinct from the interactions in apo-forms), and/or Ni site and protein rearrangements in one or both protein partners. The complexities of the Ni,Zn-HypA interaction with apo-UreE₂ were partially clarified by experiments performed under conditions where the Ni sites in both proteins are saturated. Since HypA has been hypothesized to bind and then transfer Ni²⁺ to UreE₂,^{22, 31} one anticipated outcome was that the HypA•UreE₂ complex would not form with Ni-UreE₂, or that the protein-protein affinities would be reduced. Unexpectedly, the interaction of the HypA with UreE₂ is tighter by at least two-orders of magnitude under saturating Ni²⁺ concentrations, as compared to the apo protein-protein interactions (Figure 4 and Table 2). The pH dependency indicates that the overall formation of complex is about 4.5-fold tighter under acidic conditions. In addition, SEC-MALS showed that the complex stoichiometry under these conditions (1 HypA: 1 UreE₂) was not affected by pH (Figure 6). The complex was separated chromatographically and analyzed for Ni and Zn content, which showed that Ni,Zn-HypA•UreE₂ maintained a Ni:Zn ratio close to 1:1 at pH 7.2 and 6.3. The results are consistent with the formation of a HypA•UreE₂ protein complex containing a single tight Ni-binding site, since the Ni content is equal to the Zn content of the HypA protein present in the complex. The fact that the Ni,Zn-HypA•UreE₂ complex contains a novel tight Ni-binding site suggests that it is located at the HypA•UreE₂ interface and is composed of ligands from both proteins in the complex. This hypothesis is supported by the loss of the high affinity Ni binding event when the native HypA Ni-site in the complex was altered by substituting with the L2*-HypA variant (Figure 5).

A long-standing hypothesis for the role of HypA in Ni trafficking in *H. pylori* is that HypA acquires and then delivers Ni²⁺ to UreE₂ (Figure 1, Step 1A° and 1B°).^{20, 22} Prior *in vitro* and *in vivo* experiments performed to elucidate the proposed handoff of Ni from HypA to UreE₂ yielded ambiguous results indicating that UreE₂ was able to acquire Ni²⁺ directly as well as from HypA.²⁰ In support of the handoff mechanism, transfer of Ni from GS-HypA to UreE₂ was demonstrated with Ni-specific cross-linking followed by separating the protein complex under denaturing conditions. However, the GS-HypA•UreE₂ complex does not have the high-affinity Ni-binding site, and UreE₂ has the tighter Ni-binding affinity of the two isolated proteins.³¹ Another feature of the hypothesis is that Ni-UreE₂ may no longer interact with apo,Zn-HypA following the transfer, or that the interaction would weaken significantly (Figure 1, Step 1B*). In fact, the results reported here demonstrate that the Ni,Zn-HypA•UreE₂ complex remains intact in SEC (Figure 6) at sub-micromolar

concentrations, indicating that neither Ni^{2+} nor Zn-HypA are likely to dissociate from the complex without further disruptions.

These results are more consistent with an alternative mechanism wherein HypA and UreE₂ act as co-metallochaperones to target Ni to the urease maturation pathway by forming the Ni,Zn-HypA•UreE₂ complex that contains a unique high-affinity Ni-binding site and is more stable to hydrolysis than is *H. pylori* UreE₂ (Figure 7). By stabilizing UreE₂, the complex provides another level of control for urease maturation. Unlike UreE in other urease-containing organisms, the short C-terminal Ni-binding motif in *H. pylori* UreE has a lower Ni-binding capacity¹¹ and tends to be disordered¹² or degraded.¹⁸ With the sole Ni^{2+} binding site at the dimer interface in *H. pylori* UreE₂, even the degradation/instability of the C-terminal Ni-binding motif in just one subunit in the UreE₂ would hamper its ability to retain Ni^{2+} and form a complex with HypA. Given the high level of expression of apo-urease in *H. pylori*, the low Ni-binding capacity in *H. pylori* UreE₂ provides a mechanism for preventing over-activation of urease.⁴¹ Thus, one putative role for requiring HypA¹⁵ in the co-chaperone complex is to stabilize UreE₂, particularly under acid shock conditions where urease activity is critical for *H. pylori* survival. Indeed, acid-induced up-regulation of *hypA* has been previously reported.^{36, 42}

Disruption of the stable Ni,Zn-HypA•UreE₂ complex seems necessary in order to release Ni^{2+} to the urease maturation pathway. UreG, a urease accessory protein with GTPase activity,^{21, 23, 41} is known to directly interact with UreE under various conditions (Supporting Information, Figure S6).^{10, 22, 27, 29, 43} However, given the relatively weak affinity of UreG for Ni^{2+} ($K_d = 10 \mu\text{M}$; $N = 1.8$)²⁸ and that HypA can outcompete UreG for binding to UreE₂ (Figure S6, Step 2B*),²⁷ additional cofactors must be involved. For example, the binding of GTP or its analogues (GTP γ S) triggers UreG dimerization,^{22, 23} which can disrupt the GS-HypA•UreE₂ complex in favor of the formation of a UreE₂•UreG₂ complex in the presence of Mg and GTP *in vitro* (Figure S6, Step 2A°).³¹ For *in vitro* urease maturation, the release of Ni^{2+} from Ni,Zn-HypA•UreE₂ complex might involve the formation of a UreE₂•UreG₂ complex (Figure S6, Step 2A*),²³ which also disrupts the Ni,Zn-HypA•UreE₂ complex (Figure S6, Step 2A°),²² or might possibly proceed via formation of a Ni,Zn-HypA•UreE₂•UreG₂ ternary protein complex, which has not been observed thus far. Whereas both Ni-UreE₂ and the Ni,Zn-HypA•UreE₂ complex are possible sources of Ni^{2+} for urease maturation, the latter is more stable and likely the more prevalent Ni^{2+} source.

The formation of a tight Ni complex in Ni,Zn-HypA•UreE₂ might commit Ni to the urease maturation pathway, as opposed to hydrogenase maturation. The requirement of GTP-bound UreG₂ for disrupting the Ni,Zn-HypA•UreE₂ complex²² would enhance the fidelity of Ni delivery to the urease maturation pathway. The results of this study, along with previous studies,^{20, 22, 27} suggest that HypA and UreE₂ act as co-metallochaperones, offering both tight Ni-coordination and fidelity of release for Ni delivery to apo-urease in *H. pylori*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Ni	nickel
Zn	zinc
ITC	isothermal titration calorimetry
SEC-MALS	size exclusion chromatography with multi-angle light scattering
ICP-OES	inductively coupled plasma-optical emission spectroscopy
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
K_d	apparent dissociation constant
μM	micromolar; μM , nanomolar
GTP	guanosine triphosphate

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Figure 1.

Ni trafficking pathways (labeled in red) involved in *H. pylori* for urease maturation, where Step 1 describes HypA and UreE₂ interactions, culminating in the delivery of Ni to downstream processes (Step 2 describes interactions with UreG, and Step 3 describes interactions with UreH, UreF, and apo-Urease). Equilibria depicted in black were studied *in vitro* in this work, where Steps 1A described Ni interactions with metallochaperones and 1B described protein-protein interactions between metallochaperones. Dashed arrows represent sub-micromolar apparent dissociation constants (K_d), and bold arrows indicate high affinity binding (with K_d at or below nM).

Figure 2.

ITC binding isotherms for titrations of HypA (left) and UreE₂ (right) with Ni²⁺. Data are shown in color and fits as black lines. Titrations were performed at pH 7.2 (blue) and 6.3 (green) and isotherms were graphed to the same y-scale for visual comparison. Insets show properly scaled isotherms for Ni²⁺ binding to HypA. The binding curves for Ni²⁺ binding to HypA or UreE₂ were fitted with OneSite models. See Materials/Experimental Details in the supporting information for detailed fitting procedures and Figure S1 for raw ITC thermograms.

Figure 3.

ITC isotherms from titrations of apo-UreE₂ with apo,Zn-HypA (left) and Ni,Zn-HypA (right). Representations of each titration are shown above the curves. Titrations were performed at pH 7.2 (blue) and 6.3 (green). Data are shown in colored symbols and fits in black lines. Titration curves were graphed to the same y-scale for visual comparison. Data were fitted to OneSite or TwoSites models as described in the text. See Materials/Experimental Details in the supporting information for detailed fitting procedures and Figure S1 for raw ITC thermograms.

Figure 4.

Fluorescence binding curves from titrations of NHS640-UreE₂ with apo,Zn-HypA (left) and Ni,Zn-HypA (right). Titrations were performed at pH 7.2 (blue) and 6.3 (green).

Representations of each titration are shown above the binding curves, where fluorophores are represented as yellow stars. Titrations with apo,Zn-HypA as the titrant (open squares) were performed in buffer without Ni, and titrations with Ni,Zn-HypA as the titrant (filled squares) were performed with 1 μM Ni²⁺ in the buffer. Fluorescence data (colored symbols) were fitted (black lines) with the mass action function for the binding of apo,Zn-HypA to NHS640-UreE₂ at both pH values, and for Ni,Zn-HypA binding to NHS640-UreE₂ at pH 7.2, assuming 1:1 binding. The Hill Equation was used to fit the Ni,Zn-HypA binding to NHS640-UreE₂ at pH 6.3. See Materials/Experimental Details in the supporting information for detailed fitting procedures.

Figure 5.

ITC binding isotherms for titrations of the apo,Zn-WT-HypA•UreE₂ (right) and apo,Zn-L2*-HypA•UreE₂ (left) protein complexes with Ni^{2+} . Data are shown in color and fits as black lines. Titrations were performed at pH 7.2 (blue) and 6.3 (green) and isotherms were graphed to the same y-scale for visual comparison. The fits (black lines) show the loss of the tighter binding event when the L2*-HypA variant is used to form the protein complex. See Materials/Experimental Details in the supporting information for detailed fitting procedures and Figure S1 for raw ITC thermograms.

Figure 6. Size-exclusion chromatographs of Ni,Zn-HypA (gray lines), UreE₂ (cyan lines), and a 3:1 mixture of Ni,Zn-HypA with UreE₂ (blue or green line) at pH 7.2 (top panel) and at pH 6.3 (bottom panel). The deconvoluted molecular masses from MALS analyses are graphed on the center of each peak. Representations of the individual species involved are shown below each set of peaks. See Materials/Experimental Details in the supporting information for detailed analysis method.

Figure 7.

Degradation of the UreE₂ at 20°C was monitored at pH 7.2 (left) and 6.3 (right) using SDS-PAGE (upper panels), in the presence and absence of one molar equivalent of apo,Zn-HypA and two molar equivalents of Ni²⁺, as indicated at the bottom of the gel. The molecular weight positions of HypA and UreE [separated into full-length peptide (UreE) and degraded peptides (UreE C)] are marked with arrows. The decrease in full-length UreE peptide over time (lower panels) shows the average ± standard deviation from three replicates experiments.

Table 1.Summary of ITC results from Ni titrations¹

Protein	pH 7.2		pH 6.3		Ref.
	K_d (M)	N (Sites)	K_d (M)	N (Sites)	
apo,Zn-HypA	$(9.7 \pm 0.3) \times 10^{-7}$	0.94 ± 0.04	$(1.6 \pm 0.8) \times 10^{-6}$	0.92 ± 0.21	this work, ¹⁸
apo,Zn-L2*-HypA	$(5.9 \pm 1.2) \times 10^{-5}$	0.39 ± 0.07	ND ²	ND ²	¹⁸
apo-UreE ₂	$(6.7 \pm 1.5) \times 10^{-8}$	0.71 ± 0.12	$(5.3 \pm 2.5) \times 10^{-8}$	0.56 ± 0.01	this work, ¹⁸
³ apo,Zn-HypA•UreE ₂	(1) $(1.9 \pm 1.6) \times 10^{-10}$	0.54 ± 0.05	$(3.1 \pm 1.9) \times 10^{-9}$	0.52 ± 0.01	this work
	(2) $(2.9 \pm 2.3) \times 10^{-7}$	1.2 ± 0.1	$(7.2 \pm 2.1) \times 10^{-7}$	0.37 ± 0.04	
³ apo,Zn-L2*-HypA•UreE ₂	$(1.7 \pm 0.2) \times 10^{-8}$	0.49 ± 0.002	$(2.7 \pm 0.5) \times 10^{-8}$	0.48 ± 0.003	this work

¹ Results represent the average $\pm$ standard deviation from three or more independent titrations unless otherwise noted. See Methods for detailed fitting procedures.

² ND = not determined

³ Result represents the average $\pm$ range from two independent titrations.

Table 2.Summary of HypA titrations of UreE₂¹

HypA	pH 7.2		pH 6.3		Ref.
	K_d (M)	N (Sites)	K_d (M)	N (Sites)	
ITC					
apo,Zn-HypA	$(9.0 \pm 4.4) \times 10^{-7}$	1.4 ± 0.2	$(1.2 \pm 0.3) \times 10^{-6}$	1.4 ± 0.1	this work, ¹⁸
apo,Zn-L2*-HypA	$(6.9 \pm 1.1) \times 10^{-7}$	1.6 ± 0.2	$^2_{\text{ND}}$	$^2_{\text{ND}}$	18
Ni,Zn-HypA	$(1.8 \pm 0.1) \times 10^{-7}$	1.1 ± 0.1	$(3.6 \pm 3.0) \times 10^{-7}$	1.0 ± 0.1	this work
	$(6.4 \pm 4.0) \times 10^{-10}$	0.50 ± 0.09	$(6.4 \pm 2.5) \times 10^{-10}$	0.52 ± 0.06	
Fluorescence (NHS640-UreE ₂)					
apo,Zn-HypA	$(2.7 \pm 0.5) \times 10^{-6}$	1:1	$(1.8 \pm 0.2) \times 10^{-6}$	1:1	this work
$^3\text{Ni,Zn-HypA}$	$(2.0 \pm 0.9) \times 10^{-8}$	1:1	$(4.5 \pm 2.6) \times 10^{-9}$	cooperative binding	this work

¹Results reported are the average $\pm$ standard deviation of three or more independent titrations. ITC data were fitted with Microcal analysis for One- or TwoSites models. Fluorescence binding curves were fitted with mass action function assuming 1:1 binding or with the Hill Equation indicated as cooperative binding. See Methods for fitting details.

²ND = not determined.

³Measured under Ni-saturating conditions (with 1 μM Ni²⁺ in the buffer).