Relationship between Ni(II) and Zn(II) Coordination and Nucleotide Binding by the Helicobacter pylori [NiFe]-Hydrogenase and Urease Maturation Factor HypB*

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Background: Helicobacter pylori HypB (HpHypB) is a metal-regulated GTPase essential for the biosynthesis of [NiFe]-hydrogenase and urease. Nickel binding to HpHypB is altered upon nucleotide binding, an effect not observed with zinc.

Results: Metal coordination and the GTPase cycle of HpHypB are intimately linked.

Conclusion: These data suggest that HpHypB contributes to metal fidelity in [NiFe]-hydrogenase and urease biosynthesis.

The pathogen Helicobacter pylori requires two nickel-containing enzymes, urease and [NiFe]-hydrogenase, for efficient colonization of the human gastric mucosa. These enzymes possess complex metallocenters that are assembled by teams of proteins in multistep pathways. One essential accessory protein is the GTPase HypB, which is required for Ni(II) delivery to [NiFe]-hydrogenase and participates in urease maturation. Ni(II) or Zn(II) binding to a site embedded in the GTPase domain of HypB modulates the enzymatic activity, suggesting a mechanism of regulation. In this study, biochemical and structural analyses of H. pylori HypB (HpHypB) revealed an intricate link between nucleotide and metal binding. HpHypB nickel coordination, stoichiometry, and affinity were modulated by GTP and GDP, an effect not observed for zinc, and biochemical evidence suggests that His-107 coordination to nickel toggles on and off in a nucleotide-dependent manner. These results are consistent with the crystal structure of HpHypB loaded with Ni(II), GDP, and P0, which reveals a nickel site distinct from that of zinc-loaded Methanococcus jannaschii HypB as well as subtle changes to the protein structure. Furthermore, Cys-142, a metal ligand from the Switch II GTPase motif, was identified as a key component of the signal transduction between metal binding and the enzymatic activity. Finally, potassium accelerated the enzymatic activity of HpHypB but had no effect on the other biochemical properties of the protein. Altogether, this molecular level information about HpHypB provides insight into its cellular function and illuminates a possible mechanism of metal ion discrimination.

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This article contains supplemental Table S1.

The atomic coordinates and structure factors (code 4LPS) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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Helicobacter pylori is a Gram-negative, microaerophilic human pathogen responsible for a multitude of medical conditions, including chronic gastric inflammation, ulcers, and precursor lesions to gastric cancer (1, 2). Critical to the ability of H. pylori to colonize the human gastric mucosa are two Ni(II)-containing enzymes: urease and [NiFe]-hydrogenase (3–5). Urease is responsible for preventing dramatic drops in the cytosolic pH by catalyzing the hydrolysis of urea to produce buffering ammonia and bicarbonate (6–8). [NiFe]-hydrogenase provides H. pylori with an energy source by catalyzing the oxidation of molecular hydrogen, a by-product of carbohydrate fermentation by other gut microorganisms (4, 9). Both enzymes require multiple dedicated accessory proteins for biosynthesis of their intricate metallocenters. The H. pylori genome contains genes encoding the UreEFGH and HypABCDEF accessory proteins responsible for the maturation of urease and [NiFe]-hydrogenase, respectively (10). Furthermore, H. pylori with gene-directed mutations in hypA or hypB were deficient in both enzymatic activities, demonstrating that HypA and HypB bridge the two pathways (11). The production of functional enzymes in the mutant strains was at least partially restored by the addition of extra nickel to the growth media, suggesting that these proteins are involved in Ni(II) delivery during the maturation of the metalloenzymes (11).

The biosynthesis of the active site of [NiFe]-hydrogenase proceeds in two main stages with the iron, along with its carbon monoxide and cyanide ligands, incorporated first into the large subunit of the hydrogenase precursor protein (12, 13). Next, HypA and HypB are believed to cooperate to insert the nickel ion (14–16) in a process that requires the GTPase activity of HypB (17–20) and that is aided by SlyD in some organisms (21). HypA can form a complex with HypB and SlyD in the absence of the hydrogenase large subunit (22–26), and there is evidence that HypA docks this preassembled nickel insertion complex onto the [NiFe]-hydrogenase precursor protein (26). Furthermore, it has been proposed that HypA is responsible for steering Ni(II) to either the urease or hydrogenase maturation pathway (27).
There are numerous HypB homologs with varying metal binding abilities (25, 28–30), but one common trend is that all bacterial HypB homologs possess a metal-binding site located in the C-terminal part of the protein between two of the canonical GTPase motifs (21). Mutation of several of the conserved residues that serve as metal ligands in Escherichia coli HypB (EcHypB)2 abrogates hydrogenase production in this organism, indicating the critical role of this metal-binding site of HypB during metallocenter assembly (31). This is the only metal site identified in H. pylori HypB (HpHypB) that can bind one nickel ion with a $K_D$ of 150 nM (30). However, zinc binds with an affinity that is about 2 orders of magnitude tighter, prompting speculation about the identity of the physiologically relevant metal (30). The only crystal structure of metal-loaded HypB is of the protein from Methanothermococcus janmashii, which revealed a dinuclear asymmetric zinc site at the interface of a GTPyS-bound protein dimer (32). The observation that Zn(II) binding to HpHypB abolishes any detectable GTPase activity whereas Ni(II) binding has a more subtle impact prompted speculation that this metal site serves a regulatory role during hydrogenase maturation (30), but little was known how these key cofactors of HypB affect each other.

In this study, we investigated the relationship between metal binding and the GTPase cycle of HpHypB. Nucleotide altered the nickel, but not zinc, stoichiometry and modulated the affinity of the HpHypB-nickel complex. Structural analysis of nucleotide-loaded HpHypB revealed the first picture of a Ni(II)-loaded HypB protein in which a single Ni(II) ion is bound in a tetrathiolate square planar geometry at the interface of two HpHypB monomers, a coordination environment distinct from that predicted in the absence of nucleotide (28, 30–32). In support of this switch in metal coordination, biochemical examination of His-107 suggested that it serves as a nickel-binding residue only in the nucleotide-free protein. Finally, a key component of metal-mediated inhibition of GTP hydrolysis is Cys-142, a metal ligand that is donated from the signal-transducing Switch II GTPase motif. Altogether, our results demonstrate that this metal site serves a regulatory role during hydrogenase maturation (30), but little was known how these key cofactors of HypB affect each other.

### EXPERIMENTAL PROCEDURES

**Materials**—Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs. Primers were purchased from Sigma Genosys. All chromatography media were from GE Healthcare. Kanamycin, tris(2-carboxyethyl)phosphine (TCEP), isopropyl β-D-1-thiogalactopyranoside, and EDTA were purchased from BioShop (Toronto, Ontario, Canada). Nickel chloride salt (at minimum 99.9% pure) was purchased from ADI. nickel chloride salt (at minimum 99.9% pure) was purchased from ADI. All other reagents were analytical or molecular biology grade. Electronic absorption measurements, unless otherwise noted, were conducted on an Agilent 8453 spectrophotometer with a 1-cm path length. All fluorescence experiments were conducted on a J Y HORIBA Fluorolog-3 spectrophuometer. The buffers for all metal assays were treated with Chelex-100 (Bio-Rad) to minimize trace metal contamination. All solutions were prepared with Milli-Q water (18.2 MΩ-cm resistance; Millipore).

**HpHypB Expression and Purification**—The construction of the WT HpHypB-pET24b expression vector was described previously (30). The H107A and C142S mutations were introduced into the HpHypB-pET24b construct by QuikChange PCR mutagenesis (Stratagene) with Pfu polymerase using the following primers: HpHypB C142S forward, 5′-CTGAGGGAATT TGGTTCCTCCCCAGCTATAATCTAG-3′; HpHypB C142S reverse, 5′-CTAGATTAGCTTGGAGGGAACCAATTTCCCCACG-3′; HpHypB H107A forward, 5′-CCACCCGC-GAAGCATGCgcTTTGGAAGCGAGC-3′; HpHypB H107A reverse, 5′-AGTCTGCTTTCAAAGCATGGTTCCGCGGT-3′. The template strand was subsequently digested with DpnI. The parent HpHypB-pET24b and mutant plasmids were transformed into NEB Turbo E. coli competent cells (New England Biolabs) and isolated by using the Fermentas GeneJet Plasmid Miniprep kit. All plasmids were sequenced (ACGT Corp., Toronto, Ontario, Canada) in the forward and reverse directions by using the T7 promoter and terminator primers. The proteins were overexpressed and purified as reported previously (30). A sample of each protein was sent for electrospray ionization mass spectrometry (Department of Chemistry, University of Toronto), and the determined molecular masses of the WT, H107A, and C142S proteins corresponded to their calculated molecular masses (MM): WT HpHypB, MM$_{obs}$ = 27,179.0 Da, MM$_{calc}$ = 27,179.2 Da; C142S HpHypB, MM$_{obs}$ = 27,163.0 Da, MM$_{calc}$ = 27,163.1 Da; H107A HpHypB, MM$_{obs}$ = 27,113.0 Da, MM$_{calc}$ = 27,113.1 Da.

**Preparation of Proteins**—Reduced apoprotein was produced by incubating HpHypB with 12.5 mM EDTA and 30 mM TCEP in a Coy anaerobic glovebox at 4 °C for 48 h. The TCEP and EDTA were removed by passing the protein sequentially over two PD-10 gel filtration columns (GE Healthcare) equilibrated with protein buffer (25 mM HEPES, pH 7.6 and 100 mM NaCl) in the glove box. For experiments in buffer containing 100 mM NaCl, the columns were equilibrated with 25 mM HEPES, pH 7.6 and 100 mM NaCl. The protein concentrations were calculated by using the extinction coefficient of 7,450 M$^{-1}$ cm$^{-1}$ for both WT and mutant HpHypB at 280 nm in 4 M guanidinium HCl and 25 mM EDTA (33). The absence of any metal bound to the protein was confirmed by a 4-(2-pyridylazo)resorcinol (PAR) assay (34) in which 10–20 μM protein was denatured with 4 M guanidinium HCl and 50 μM PAR was added to the sample. The absorbance at 500 nm, due to the formation of the (PAR)·Me(II) complex, was monitored and compared with a standard curve prepared with 50 μM PAR in 4 M guanidinium HCl and known metal concentrations. The free thiol content of

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2. The abbreviations used are: EcHypB, E. coli HypB; HpHypB, H. pylori HypB; MjHypB, M. jannashii HypB; TCEP, tris(2-carboxyethyl)phosphine; GDNP, guanosine 5′-[β,γ-imido]triphosphate; GTPγS, guanosine 5′-O-[(γ-thio) triphosphate; PAR, 4-(2-pyridylazo)resorcinol; MF2, Mag-Fura-2; GDP-BODIPY, guanosine 5′-diphosphate BODIPY FL F12′-(or 3′)-O-(N/2-aminoethy) urethane); GTP-BODIPY, guanosine 5′-triphosphate BODIPY FL F12′-(or 3′)-O-(N/2-aminoethy)urethane; r.m.s.d., root mean square deviation; LMCT, ligand-to-metal charge transfer; MM, molecular mass.
the proteins was measured via reaction of the protein with 5,5'-dithiobis(2-nitrobenzoic acid) in the presence of 6 M guanidinium HCl and 1 mM EDTA. β-Mercaptoethanol was used as a standard, and the absorbance of the 5-mercapto-2-nitrobenzoic acid product was measured at 412 nm. Protein samples were greater than 90% reduced after treatment with TCEP.

Metal Binding and Stoichiometry—Individual samples containing 20 μM WT, C142S, or H107A apo-HpHypB in protein buffer and varying concentrations of NiCl₂ were prepared in the glove box and incubated overnight at 4 °C. The electronic absorption spectrum was monitored between 250 and 500 nm and corrected by background subtraction at 600 nm. In samples containing nucleotide, 5 mM MgCl₂ and 100 μM GDP or GDPNP were also included. Metal stoichiometry experiments were conducted by incubating 120 μM apo-HpHypB with either 600 μM NiCl₂ or 500 μM ZnSO₄ overnight at 4 °C in the glove box. For samples containing additional ligands, 5 mM MgCl₂, 500 μM GDP, and/or 500 μM GDPNP were also included. Excess metal and ligand were removed by passing the protein through either a PD-10 or G-10 gel filtration column (GE Healthcare) equilibrated with protein buffer in the glove box. The protein concentrations were calculated as described above. To confirm no interference from nucleotide, protein concentrations were verified with a BCA assay (Thermo Scientific) equilibrated with protein buffer in the glove box.

To determine the nickel affinity of WT HpHypB, competition experiments were prepared by incubating 10 μM WT or mutant HpHypB together with 10 μM MF2 and varying amounts of NiCl₂ in protein buffer. The samples were incubated overnight at 4 °C in the glove box. The absorbance of MF2 at 366 nm was monitored using electronic absorption spectroscopy, and the data were analyzed by using DYNASTAT (37) using a custom DYNAFIT script that describes the competition between the protein and MF2 for the metal.

WT HpHypB Ni(II) Affinity in the Presence of Magnesium and Nucleotide—The affinity of WT HpHypB for Ni(II) in the presence of MgCl₂ and GDP or GDPNP was also determined using MF2. Although MF2 was originally developed as a magnesium-sensitive fluorescent dye, the MF2-Mg(II) emission spectrum at 505 nm upon excitation at 335 nm decreases as the Mg(II) is displaced by the tighter binding Ni(II) (see Fig. 1C, inset). This differential response to Ni(II) in the presence of Mg(II) allows for the use of MF2 as a competitor. The affinity of MF2 for Ni(II) in the presence of 5 mM MgCl₂, 500 μM GDP, or 500 μM GDPNP was determined by incubating 50 nM MF2 with the corresponding ligand and varying NiCl₂ concentrations for at least 2 h. The decrease of the emission spectrum at 505 nm upon excitation at 335 nm was monitored via fluorescence, and the data were analyzed as described above for MF2 in the absence of magnesium or nucleotide. In the presence of 5 mM Mg(II), the MF2 Ni(II) apparent Kₐ was determined to be (7 ± 2) × 10⁻⁷ M. Similarly, control titrations yielded apparent MF2 Ni(II) Kₐ values of 1.5 ± 0.3 and 1.8 ± 0.3 μM in the presence of GDPNP and GDP, respectively.

Competition experiments were prepared by incubating 10 μM WT apo-HpHypB together with 5 μM MF2 and varying amounts of Ni(II) in the presence of 5 mM MgCl₂ and 500 μM GDP or GDPNP. The samples were incubated overnight at 4 °C in the glove box, the fluorescence decrease at 505 nm upon excitation at 335 nm was monitored, and the data were analyzed using DYNASTAT (37) using a custom DYNAFIT script that describes the competition between the dimeric protein and MF2 for the metal.

**Mutant HpHypB Nickel Affinity**—Weakened metal binding by the H107A and C142S HpHypB mutants permitted the use of the Ni(II)-protein electronic absorption signal to measure the nickel affinities. For nickel binding to apo-C142S and apo-H107A HpHypB, 5 μM (for C142S + GDP and H107A + GDP) or 10 μM mutant protein (for all other nickel titrations) was incubated overnight at 4 °C in the glove box in protein buffer with 5 mM MgCl₂, with increasing concentrations of NiCl₂. The electronic absorption spectrum was monitored between 250 and 500 nm and corrected by background subtraction at 600 nm. In samples containing nucleotide, 5 mM MgCl₂ and 500 μM GDP or GDPNP were also included. The apparent Kₐ was calculated by determining the fractional saturation, r, and free
nickel concentration, \([\text{Ni(II)}]_{\text{free}}\), by using Equations 4 and 5, respectively.

\[
 r = \frac{[\text{HypB-Ni(II)}]}{[\text{HypB}]_{\text{total}}} = \frac{A_{\text{obs}} - A_{\text{min}}}{A_{\text{max}} - A_{\text{min}}} \quad (\text{Eq. 4})
\]

\[
 [\text{Ni(II)}]_{\text{free}} = [\text{Ni(II)}]_{\text{total}} - (r \times [\text{HypB}]_{\text{total}}) \quad (\text{Eq. 5})
\]

where \([\text{HypB-Ni(II)}]\) is the concentration of protein bound to Ni(II), \([\text{HypB}]_{\text{total}}\) is the total protein concentration, \(A_{\text{obs}}\) is the absorbance at 340 nm for a given Ni(II) concentration, \(A_{\text{min}}\) is the absorbance at 340 nm for apo-HypB, \(A_{\text{max}}\) is the absorbance at 340 nm upon saturation, and \([\text{Ni(II)}]_{\text{free}}\) is the total Ni(II) concentration added to the sample. The resulting values were plotted as \(r\) versus \([\text{Ni(II)}]_{\text{free}}\) and the data were fit to the Hill equation (Equation 3) as described above except that \(n\) was allowed to vary.

**HypB Zincon Competition for Zinc**—To estimate the Zn(II) \(K_r\) of WT and mutant HpHypB, the competitor zincon was selected because of its ability to form a 1:1 complex with Zn(II) with a reported \(K_r\) of 12.7 \(\mu\)M (38, 39). Stocks of zincon were prepared in Milli-Q water. The affinity of zincon for Zn(II) was verified under our experimental conditions by titrating 400 \(\mu\)M zincon in protein buffer with increasing amounts of ZnSO\(_4\). The absorbance at 620 nm was monitored using a 10-cm-path length cuvette and a GBC Cintra 404 spectrophotometer. The data were analyzed by using Equations 4 and 5 and fit to the Hill equation (Equation 3) with \(n = 1\). Under our buffer conditions, the \(K_r\) of zincon for Zn(II) was determined to be 7 ± 1 \(\mu\)M in agreement with previously reported \(K_r\) values (35, 38, 39). Competition experiments were prepared by incubating 20 \(\mu\)M apo-WT or mutant HpHypB with 140 \(\mu\)M zincon and various amounts of ZnSO\(_4\). The samples were incubated overnight at 4 °C in the glove box. The absorbance of zincon at 620 nm was monitored using a 2-mm-path length cuvette.

**WT HpHypB Zinc Affinity**—The affinity of MF2 for zinc under our buffer conditions in the absence of magnesium or nucleotide was measured as described above for MF2 and nickel and was determined to be 80 ± 17 \(\mu\)M in agreement with previously published values (30, 36). The affinity of WT HpHypB for Zn(II) in protein buffer without magnesium or nucleotide was determined by using MF2 as described above for the Ni(II) competitions.

MF2 can be used to monitor Zn(II) binding in the presence of Mg(II) by taking advantage of the different excitation wavelength maxima for the Zn(II)- and Mg(II)-bound MF2 species as described previously by Simons (40). The Mg(II)-MF2 complex features an excitation wavelength maxima at 335 nm as opposed to 321 nm for Zn(II)-MF2 when fluorescence is measured at 505 nm (see Fig. 1D, inset) (40). Thus, by monitoring the increasing fluorescence at 505 nm upon excitation at 321 nm while in the presence of 5 \(\mu\)M MgCl\(_2\), MF2 can be used as a Zn(II) reporter. The affinity of MF2 for Zn(II) in the presence of magnesium and nucleotide was determined in a similar manner as for Ni(II) except for the following differences: 20 \(\mu\)M MF2 was used for the titrations, the fluorescence at 505 nm was monitored upon excitation at 321 nm, and the fractional saturation was calculated by using Equation 6.

\[
 r = \frac{[\text{MF-Zn(II)}]}{[\text{MF}]_{\text{total}}} = \frac{F_{\text{obs}} - F_{\text{min}}}{F_{\text{max}} - F_{\text{min}}} \quad (\text{Eq. 6})
\]

where in this case \(F_{\text{obs}}\) is the fluorescence intensity at 505 nm for a given Zn(II) concentration, \(F_{\text{max}}\) is the fluorescence at 505 nm upon saturation of MF2 with Zn(II), and \(F_{\text{min}}\) is the fluorescence at 505 nm of apo-MF2. In the presence of 5 \(\mu\)M Mg(II), the MF2 Zn(II) apparent \(K_r\) was determined to be \((2.7 ± 0.7) \times 10^{-7}\) M. Similarly, control titrations yielded apparent MF2 Zn(II) \(K_r\) values of \((5.9 ± 0.9) \times 10^{-7}\) and \((3.8 ± 0.7) \times 10^{-7}\) M in the presence of GDPNP and GDP, respectively.

**Competition Experiments**—Competition experiments were prepared by incubating 5 \(\mu\)M WT apo-HpHypB together with 5 \(\mu\)M MF2 and varying amounts of Zn(II) in the presence of 5 \(\mu\)M MgCl\(_2\) and 500 \(\mu\)M GDP or GDPNP. The samples were incubated overnight at 4 °C in the glove box, and the decrease of the fluorescence upon excitation at 321 nm was monitored at 505 nm, and the data were analyzed by using DYNAFIT (37) using a custom DYNAFIT script that describes the competition between the protein and MF2 for the metal.

**Analytical Gel Filtration Chromatography**—Samples containing 50 \(\mu\)M WT or mutant HpHypB were incubated with the desired metal or nucleotide overnight at 4 °C in the glove box. All samples contained 25 mM HEPES, pH 7.6, 100 mM KCl, and 5 mM MgCl\(_2\). Samples were loaded onto a Superdex 200 10/300 analytical gel filtration column (GE Healthcare) equilibrated with filtered and chelated 25 mM HEPES, pH 7.6, 200 mM KCl, and 5 mM MgCl\(_2\). The chromatogram was monitored at 280 nm. The column was calibrated with thyroglobulin (670 kDa), \(\gamma\)-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B\(_{12}\) (1.4 kDa) from Bio-Rad. No difference in the elution profile of these standards proteins was observed when 140 \(\mu\)M NiCl\(_2\) or ZnSO\(_4\) was added prior to gel filtration chromatography. Molecular masses were determined by plotting the log molecular masses of the standards versus the partition coefficients (\(K_r\)) where \(K_r = (V_r - V_o)/(V_e - V_o)\); \(V_o\) represents the elution volume, \(V_e\) is the void volume, and \(V_r\) is the total column volume.

**Circular Dichroism (CD) Spectroscopy**—WT and mutant HpHypB samples were prepared for CD spectroscopy by buffer exchanging the protein into 100 mM potassium or sodium phosphate buffer, pH 7.6 using a PD-10 column. The samples were then diluted to a final concentration of 10–20 \(\mu\)M, and metal or nucleotide was added and incubated overnight at 4 °C in the glove box. Protein samples with nucleotide included 5 mM MgCl\(_2\) and were incubated overnight at 4 °C in the glove box. All samples were analyzed on an Olis RSM 1000 spectropolarimeter with a capped 1-mm-path length cuvette to minimize exposure to the air. Spectra were collected at 1-nm intervals over a spectral range of 200–260 nm with an integration time of 2 s and 2400 grating lines/nm. The final spectra obtained are averages of three scans and were corrected by subtracting the background buffer signal. The observed ellipticity was converted into mean residue ellipticity ([\(\theta\)]\(_{\text{mre}}\) in \(\text{deg cm}^2 \text{ dmol}^{-1}\)) using the following formula (41).

\[
 [\theta]_{\text{mre}} = \frac{\left(\frac{MW}{N - 1}\right) \times \theta}{[\text{Protein}] \times I \times 10} \quad (\text{Eq. 7})
\]
where MW is the molecular mass of the protein in Da, N is the number of amino acids, \( \theta \) is the observed ellipticity in degrees, [Protein] is the concentration of protein in g/ml, and \( l \) is the path length.

**GTPase Assays—**GTPase activity was determined by using the malachite green assay for free phosphate adapted from Lanzetta et al. (42). A series of 400-µl samples containing 2 µM WT or H107A HpHypB (in 25 mM HEPES, pH 7.6 and 100 mM NaCl or KCl), 5 mM MgCl₂, and varying GTP concentrations were incubated at 37 °C in the glove box for 30 min. Controls containing only buffer, 5 mM MgCl₂, and the corresponding GTP concentrations were prepared alongside the protein samples and received the same treatment. After incubation, 100 µl of the phosphate detection reagent (2.6 mM malachite green, 1.5% ammonium molybdate, and 0.2% Tween 20) was added to each sample. The samples were mixed by vortexing and incubated at room temperature for 3 min after which sodium citrate was added to a final concentration of 3.5%. The samples were then mixed again, the color was allowed to develop for 30 min before placing onto a 96-well plate, and the absorbance was measured at 630 nm with a Teco Safire2 microplate reader. The amount of phosphate released was determined via a standard curve from a phosphate standard (Molecular Probes). The data were analyzed by fitting to the Michaelis-Menten equation using OriginPro 8. Samples containing metal were preincubated with the metal overnight prior to the assay (prepared as a stock of 40 µM protein with 200 µM NiCl₂ or 100 µM ZnSO₄ for WT or 500 µM NiCl₂ or 500 µM ZnSO₄ for H107A). These stocks were then diluted to a final protein concentration of 2 µM for the assay in a buffer that contained either 200 µM NiCl₂ or 100 µM ZnSO₄ for WT or 500 µM NiCl₂ or 500 µM ZnSO₄ for H107A. To ensure that the time period selected (30 min) was on the linear portion of the hydrolysis curve, WT apo-HpHypB was incubated with 950 µM GTP for various time periods, and each sample was then worked up as described above. The 30-min time period lies within the linear portion of the curve, which extends up to 60 min (data not shown).

The \( k_{cat} \)—only measurements of C142S HpHypB were conducted using 2 µM apo-C142S HpHypB with 1.4 mM GTP for 30 min at 37 °C in 25 mM HEPES, pH 7.6, 100 mM KCl, and 5 mM MgCl₂ and worked up as described above. Samples of C142S HpHypB containing metal were preincubated with the metal overnight prior to the assay (prepared as a stock of 40 µM protein with 1 mM NiCl₂ or 500 µM ZnSO₄). These stocks were then diluted to a final protein concentration of 2 µM for the assay in a buffer that contained either 1 mM NiCl₂ or 500 µM ZnSO₄.

**Nucleotide Binding to WT and Mutant HpHypB—**To determine the affinity of WT and mutant HpHypB for GDP and GTP in the presence and absence of metal, a 50 nM concentration of fluorescently labeled guanosine 5’-diphosphate BODIPY FL 2’-(or 3’)-O-(N-(2-aminoethyl)urethane), bis(triethylammonium) salt (GDP-BODIPY; Invitrogen) or guanosine 5’-triphosphate BODIPY FL 2’-(or 3’)-O-(N-(2-aminoethyl)urethane), bis(triethylammonium) salt (GTP-BODIPY; Invitrogen) was incubated for at least 2 h at 4 °C in the dark in the glove box with increasing amounts of protein. The change in the fluorescence of the BODIPY dyes excited at 488 nm was monitored at 509 nm.

**Results**

HpHypB is activated by Potassium and Inhibited by Nickel and Zinc—HpHypB possesses low GTPase activity, which prompted speculation that additional cofactors may be needed to increase the rate of GTP hydrolysis (30). A recent study of the

**Data Collection and Structure Determination—**Data were collected at beamline BM30A of the European Synchrotron Radiation Facility in Grenoble, France. Data reduction was carried out using XDS (43). To identify the nature of the metal bound to HpHypB, five x-ray diffraction data sets were subsequently collected from one monoclinic crystal at the following x-ray wavelengths: \( \lambda_{se} = 0.97969 \) Å (maximum for selenium), \( \lambda_{zn1} = 1.28616 \) Å, \( \lambda_{zn2} = 1.28616 \) Å, \( \lambda_{ni1} = 1.48627 \) Å, \( \lambda_{ni2} = 1.49162 \) Å (corresponding to both sides of maximum for zinc and nickel, respectively). The structure was solved with Phaser (44) by the molecular replacement method using as a starting model the atomic coordinates of the HypB x-ray model from M. jannaschii (32) (Protein Data Bank ID code 2HF9). Crystallographic refinements were conducted using PHENIX (45), and the three-dimensional models were examined and modified using the graphics program Coot (46). Crystallographic statistics are summarized in supplemental Table S1. Superimpositions of models and root mean square deviation (r.m.s.d.) values were calculated using the secondary structure matching tool (47). Fig. 2 was prepared with PyMOL (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC.). The Protein Data Bank code is 4LPS.
[FeFe]-hydrogenase maturation factor HydF, which is also a GTPase (49–52). Similarly, in the presence of 100 mM KCl, the metal binding affinities of WT and mutant HpHypB in the absence and presence of nucleotide were determined.

To determine the affinity of WT HpHypB for nickel, a competition between WT HpHypB and Mag-fura-2 (MF2) was established. In the case of WT protein without Mg(II) or nucleotide, the competition was monitored via electronic absorption spectroscopy. For all WT samples, fluorescence spectroscopy was used. In both cases, the resulting data were fit by using DYNAFIT with a model of one nickel binding per protein monomer (in the absence of nucleotide) or dimer (with GDPNP or GDP).

The fluorescence intensity of free BODIPY Fl nucleotide was subtracted prior to fitting the data to the Hill equation to calculate the $K_d$ as well as the Hill coefficient $n$. The Hill coefficient $n$ is the effective number of binding sites per molecule, and it indicates the cooperativity of the binding process.

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was observed (Fig. 1A) as reported previously (30). The addition of GDP or GDPNP shifted the peak to 330 and 337 nm, respectively, suggesting a change in the Ni(II) coordination sphere (Fig. 1B). This change in nickel stoichiometry was confirmed by incubating the protein with excess metal in the presence and absence of nucleotide followed by gel filtration chromatography and then metal analysis with a colorimetric assay (Table 4). In contrast, the zinc stoichiometry was unaltered regardless of the nucleotide-bound state of HpHypB (Table 4).

Competition experiments with the metallochromic indicator MF2 were used to measure the affinity of WT HpHypB for nickel in the presence of nucleotide. The apparent $K_d$ of WT HpHypB for Ni(II) in the presence of 5 mM Mg(II) was calculated to be $320 \pm 40$ nM, which is similar to the Ni(II) $K_d$ determined in the absence of Mg(II) (Table 2 and Fig. 1C). The addition of 500 $\mu$M GDPNP tightens the nickel binding, whereas GDP weakens it, resulting in a 7-fold difference in nickel affinity between the pre- and posthydrolysis states. Competition experiments with MF2 were also conducted to measure the affinity of WT HpHypB for zinc in the presence of nucleotide, yielding apparent $K_d$ values of $40 \pm 20, 48 \pm 6,$ and $120 \pm 20$ nM in the presence of Mg(II), GDP, and GDPNP, respectively (Fig. 1D).

Crystal Structure of Ni(II)-loaded HpHypB—To examine the structure of HpHypB bound to nickel, the crystal structure of Ni(II)-HpHypB was solved at a resolution of 2.0 Å (Fig. 2A and supplemental Table S1). The first 27 residues are not visible in the electron density map. One protein dimer (composed of monomers A and B) is present in the asymmetric unit, and the two monomers are related by a non-crystallographic 2-fold axis. Each monomer has a Ni(II)-centered structure of the metal ion. Each monomer from at least three independent experiments.

**TABLE 4**

| Metal added      | Additional ligands       | Metal bounda |
|------------------|--------------------------|--------------|
| Ni(II)           |                          | 0.9 ± 0.1    |
| 5 mM MgCl$_2$    |                          | 0.9 ± 0.1    |
| 5 mM MgCl$_2$, 500 $\mu$M GDP |           | 0.5 ± 0.1    |
| 5 mM MgCl$_2$, 500 $\mu$M GDPNP |            | 1.0 ± 0.3    |
| Zn(II)           |                          | 1.2 ± 0.2    |
| 5 mM MgCl$_2$    |                          | 1.0 ± 0.1    |
| 5 mM MgCl$_2$, 500 $\mu$M GDP |           | 0.9 ± 0.2    |

$^a$ The data listed are average values and S.D. of the number of metal ions per protein monomer from at least three independent experiments.
HypB adopts an α/β fold with a central seven-stranded parallel β-sheet sandwiched by α-helices, which is typical of the SIMIBI class of GTPases (54). This fold is similar to the structures of HypB from M. jannaschii (32) and Archeoglobus fulgidus (55) with an r.m.s.d. of 1.5 Å over 195 and 184 equivalent residues, respectively, considering monomers A.

The GTP analog GTPγS was included during crystallization, but two GDP and inorganic phosphates (P_i) were modeled bound to HpHypB at the homodimer interface instead. This result is most likely explained by GTPγS hydrolysis during crystal growth (56) or contamination of the analog stock. The major difference between two monomers is that the Mg(II) ion is ligated in tetrahedral geometries (called ZnA and ZnB). ZnA is coordinated by Cys-106, ZnB to complete the square planar coordination sphere of the metal (Fig. 2B). This metal coordination is different from that observed in the GTPγS-bound M. jannaschii HypB (MjHypB) structure in which two Zn(II) ions are ligated in tetrahedral geometries (called ZnA and ZnB). ZnA is coordinated by Cys-127 (corresponding to Cys-142 in HpHypB) from monomer A, Cys-95 (corresponding to Cys-106 in HpHypB) from both monomers A and B, and a water molecule (32). ZnB is only coordinated by residues belonging to monomer B: Cys-95, Cys-127, and His-96 (corresponding to His-107 in HpHypB). A water molecule completes the coordination sphere of the metal. Superposition of the HpHypB and MjHypB metal-binding sites reveals that the nickel ion and ZnA are very close, and they are both positioned at the nickel site proposed by Chan et al. (55) (Fig. 2D). The main difference in the HpHypB metal-binding site compared with ZnA in MjHypB is the movement of Cys-106 and Cys-142 from monomers A and B, adopting a square planar geometry (Fig. 2B). This metal coordination is different from that observed in the GTPγS-bound M. jannaschii HypB (MjHypB) structure in which two Zn(II) ions are ligated in tetrahedral geometries (called ZnA and ZnB). ZnA is coordinated by Cys-127 (corresponding to Cys-142 in HpHypB) from monomer A, Cys-95 (corresponding to Cys-106 in HpHypB) from both monomers A and B, and a water molecule (32). 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![Graph](image)

**FIGURE 3.** Metal binding to H107A and C142S HpHypB. A, Ni(II) was titrated into a sample of apo-H107A (black squares) and C142S HpHypB (gray squares), and the increase in absorption at 340 nm was used to calculate the fractional saturation. Data like those shown were fit to the Hill equation to yield the apparent Hill coefficient values reported in Table 2. In the presence of GDP (black triangles), the affinity of H107A for nickel increases relative to nucleotide-free mutant. The addition of GDPNP to C142S HpHypB (gray circles) does not alter the Ni(II) affinity. Inset, the difference spectra of 20 μM C142S HpHypB (black line) and 10 μM H107A HpHypB (gray line) incubated with 1 mM and 800 μM NiCl₂, respectively. The extinction coefficients were calculated on the basis of the protein concentration. B, the signal at 620 nm was monitored as 140 μM zinc was titrated with zinc in the absence (black diamonds) or presence of 20 μM WT HpHypB (black circles) or C142S HpHypB (gray squares). C, in the absence of metal, 50 μM H107A HpHypB eluted from a gel filtration column at a volume corresponding to a monomer (solid black line). The addition of 2 mM NiCl₂ (black dashed line) resulted in the formation of a very small amount of dimeric species. Incubation of H107A HpHypB with 500 μM GDP (gray dashed line) did not change the oligomeric state of the protein. However, when both 2 mM NiCl₂ and 500 μM GDP were included, the protein dimerized (solid gray line). The large peaks at 20–25 ml are due to free nucleotide. From left to right, the identities of the protein standards (and their molecular masses), denoted by the ticks at the top of the graph, are thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B₁₂ (1.4 kDa), respectively. mAU, milliabsorbance units.

than the γ-phosphate of the GTP analog. The interactions of the proteins with the ribose and the α- and β-phosphates are very similar. In the case of the guanine base, the interactions are different due to several non-conserved residues. For example, in regard to the (S/N)KXD base recognition motif, Ser-182 does not appear to interact with the nucleotide in HpHypB, whereas the corresponding residue in MjHypB, Asn-167, does. Secondary structure matching (47) revealed that there are no major differences between the G1 (P-loop/Walker A), G2 (Switch I), and G3 (Switch II/Walker B) motifs in the two HypB structures. Together, these observations suggest that the “GDP + P₃” form may be considered as analogous to the “GTP” form found in MjHypB.

Comparison of the Ni(II)-bound HpHypB structure with that of the Zn(II)-bound MjHypB revealed several slight but global rearrangements (Fig. 2C), particularly in two regions with larger r.m.s.d. values of about 2.4 Å. The first region, residues 188–203, is positioned just after the G4 (L/KKXD) motif so it is connected to guanine binding. Conformational changes within this region may modulate the stability of the HypB homodimer as it was demonstrated previously that Met-186 and Phe-190 are involved in hydrophobic contacts at the dimeric interface and play a role in dimer stability (25). The second region corresponds to residues 216–223 just after the G5 motif (the poorly conserved SAK sequence). These conformational changes impact guanine fixation, decreasing the number of direct interactions with the protein (Fig. 2E), and result in shifts of the secondary structural elements at the protein surface (Fig. 2C).

His-107 Is a Critical Ni(II) Ligand Only in the Absence of Nucleotide—The tetrathiolate nickel coordination sphere observed in the crystal structure is surprising given that previous solution studies of HypB provide evidence for at least one imidazole ligand, that of His-107 (28, 30, 31). To further investigate this residue, a H107A mutant was constructed. Electronic absorption spectroscopy revealed that the addition of nickel to apo-H107A in the absence of nucleotide resulted in an LMCT band centered around 340 nm (Fig. 3A, inset) as opposed to the 350-nm band observed in the WT protein, suggesting that removal of H107A perturbs the Ni(II) coordination site. Furthermore, in contrast to WT HpHypB, the addition of nucleotide to the mutant protein did not alter the energy of this band. Mutation of His-107 resulted in a dramatically weakened Ni(II) affinity (Table 2) by 4 orders of magnitude, suggesting that it is a required ligand in the nucleotide-free protein. However, when the protein was loaded with nucleotide, the nickel affinity increased by several orders of magnitude (Fig. 3A and Table 2). Further evidence of this increased metal binding abil-
altered GTP binding/hydrolysis (57). To test this possibility, 

Another metal-binding residue with a potential role in communicating the metal-bound state of \( \text{HpHypB} \) to the GTPase activity is Cys-142. This residue is part of the Switch II motif (Fig. 4A), which typically undergoes a reorganization coupled to GTP hydrolysis, and is thought to link effector binding to altered GTP binding/hydrolysis (57). To test this possibility, Cys-142 of \( \text{HpHypB} \) was mutated to serine, and the properties of the mutant protein were examined. This protein still bound nickel, and the electronic absorption spectrum displayed an LMCT band, suggesting that Ni(II) binding had been disrupted. As in the nucleotide-free state, C142S \( \text{HpHypB} \) features diminished Ni(II) affinity compared with WT (Fig. 3A and Table 2). The addition of GDPNP did not drastically affect the affinity of C142S for Ni(II) (Table 2 and Fig. 3A). The GDP-bound C142S mutant, however, no longer displayed any detectable LMCT bands, suggesting that Ni(II) binding had been disrupted.

To determine whether C142S still binds Zn(II) in the absence of nucleotide, a competition between C142S \( \text{HpHypB} \) and the metalochromic indicator zincon was established. In the absence of protein, metal loading of 140 \( \mu \)M zinc was completely blocked by the addition of 140 \( \mu \)M Zn(II) (Fig. 3B). Upon the inclusion of 20 \( \mu \)M WT \( \text{HpHypB} \), Zn(II) binding to zincon was not detected until more than 20 \( \mu \)M metal had been added, and 160 \( \mu \)M total Zn(II) was required to saturate the indicator (Fig. 3B). This result is consistent with stoichiometric zinc binding to WT \( \text{HpHypB} \) with a tighter affinity than that of zincon. When the same competition was conducted with 20 \( \mu \)M C142S \( \text{HpHypB} \), 160 \( \mu \)M Zn(II) was still required to achieve zincon saturation (Fig. 3B), indicating that the C142S mutant of \( \text{HpHypB} \) maintains the ability to bind Zn(II), although the initial plateau region was less prominent than for WT \( \text{HpHypB} \).

Given that the protein retains the ability to bind transition metal ions, GTPase assays were conducted on the C142S mutant to determine whether removal of this ligand disconnects the metal responsiveness in \( \text{HpHypB} \). It was not possible to generate full Michaelis-Menten curves because of the diminished GTP affinity (Table 3), so only \( k_{\text{cat}} \) measurements were performed. The \( k_{\text{cat}} \) of the apo-C142S \( \text{HpHypB} \) was \( \sim 4 \) times lower than that of apo-WT (Table 1). In contrast to the significant inhibition observed with the WT protein, the addition of zinc only caused a 2-fold decrease in \( k_{\text{cat}} \) in C142S \( \text{HpHypB} \) (Table 1). Similarly, the \( k_{\text{cat}} \) of the mutant protein decreased \( \sim 2 \)-fold in the presence of nickel.

DISCUSSION

The role of \( \text{HpHypB} \) in the biosynthesis of the nickel-containing hydrogenase enzyme in \( H. \ pylori \) requires functional GTP hydrolysis (19), and the GTPase activity of this protein is intimately linked to selective interactions with nickel and zinc. The two metals have a different impact on the structure of the protein, particularly in the nucleotide-bound states, suggesting a means to distinguish between the metal-loaded forms. This information provides insight into the mechanisms of hydrogenase biosynthesis and can be integrated into our current working model of this pathway (Fig. 4B) along with other available data.

Apo-\( \text{HpHypB} \) dimerizes upon binding stoichiometric nickel in agreement with previous studies (25, 30). When GTP binds, the metal stoichiometry decreases. The GTP hydrolysis of isolated \( \text{HpHypB} \) is very low as observed for other members of the same GTPase family (20, 35, 58, 59), and it is even weaker when Ni(II) is bound. This metal-mediated inhibition could be a means to minimize futile cycling by \( \text{HpHypB} \) until it enters into the hydrogenase biosynthetic pathway at which point one or more of the other proteins in the pathway could stimulate this activity. For example, recent studies with \( \text{EcHypB} \) and SlyD demonstrated that SlyD accelerates GTPase activity in \( \text{HpHypB} \) (60). Once in the GDP-loaded state, the Ni(II) affinity weakens compared with the GTP-bound protein, leading to the model that GTP hydrolysis could trigger nickel release to subsequent steps of the biosynthetic pathway. We propose that the difference in Ni(II) affinity between the GTP- and GDP-bound states could be further magnified due to other components of the hydrogenase pathway. In support of this model, \( E. \ coli \) HypA stimulates nickel release from the GDP-bound, but not the GTP-bound, HypB protein (61). This mechanism of action of HypB corresponds to recent observations made with UreG (62), a closely related GTPase required for urease maturation. \( H. \ pylori \) UreG binds a single nickel ion per dimer at the interface between two UreG monomers in a site homologous to that of the \( \text{HpHypB} \) (62), and Zn(II) does not have the same impact on quaternary structure (59, 62, 63). Upon hydrolysis of GTP, UreG releases Ni(II), possibly for insertion into the nascent urease enzyme (62).
nucleotide. The nickel stoichiometry measured in solution is consistent with that observed in the crystal structure of nucleotide-loaded HpHypB, which revealed a single nickel ion shared at a dimer interface. A similar decrease in metal stoichiometry was observed for E. coli YjiA (35), a member of the same G3E family of GTPases to which HpHypB belongs (54), suggesting that this impact on metal binding by nucleotide loading may be a family-wide trait. However, it is unclear whether HpHypB exists in the nucleotide-free state in the H. pylori cytoplasm based on the reported concentration of nucleotides in other Gram-negative bacteria (64).

The change in the nickel-binding site of HpHypB that is induced by nucleotide includes the loss of His-107 coordination. Previous biochemical analysis of an HpHypB double mutant suggested that this could affect metal binding and nucleotide hydrolysis. However, the specific role of each residue in metal coordination and nucleotide hydrolysis requires further investigation.

Amino acid alignment of HpHypB with homologs. The metal-binding cysteines and histidine are indicated with asterisks and a dot, respectively. The specific strains are as follows: H. pylori 26695, Helicobacter hepaticus ATCC 51449, M. jannaschii DSM 2661, E. coli K12 substrain MG1655, Bradyrhizobium japonicum USDA110, and Rhizobium leguminosarum. All sequences were retrieved from the NCBI database and aligned by using ClustalW (68).

FIGURE 4. HypB sequence alignment and proposed model of Ni(II) versus Zn(II) discrimination by HpHypB. A, amino acid alignment of HpHypB with homologs. The metal-binding cysteines and histidine are indicated with asterisks and a dot, respectively. The specific strains are as follows: H. pylori 26695, Helicobacter hepaticus ATCC 51449, M. jannaschii DSM 2661, E. coli K12 substrain MG1655, Bradyrhizobium japonicum USDA110, and Rhizobium leguminosarum. All sequences were retrieved from the NCBI database and aligned by using ClustalW (68). B, working model for the different states of HpHypB. Nickel binds to the nucleotide-free protein in a 1:1 complex and induces protein dimer formation. Upon GTP binding, the stoichiometry decreases to a single Ni(II) ion per dimer. GTP hydrolysis may be stimulated by other components of the maturation pathway, such as SlyD or the hydrogenase precursor protein. The GDP-bound HpHypB possesses weaker Ni(II) affinity, which could lead to Ni(II) donation that is further assisted by other components of the maturation pathway, such as HypA. If Zn(II) binds instead, the protein is incapable of GTP hydrolysis. Other structural changes imposed on the protein by the differential coordination of zinc versus nickel may modulate protein-protein interactions. These effects of zinc may prevent the transfer of Zn(II) to any downstream steps in the maturation of the enzyme metallocenter.
structures are superimposable, and the GDP-

in the absence of nucleotide. In contrast, the crystal structure of nucleotide-loaded hypB revealed a tetrathiolate coordination site around the nickel ion with Cys-106 and Cys-142 serving as metal ligands. Furthermore, analysis of H107A hypB revealed that this substitution had a much larger impact on nickel binding in the nucleotide-free protein, consistent with a nucleotide-mediated change in nickel coordination. Loading the mutant protein with either GDP or GDPNP resulted in significantly stronger Ni(II) binding, although the affinity was still at least 10 times weaker than that of WT hypB in the presence of nucleotide. This latter result suggests that although His-107 does not play a critical role in nickel binding to the nucleotide-loaded states it may still exert some influence. Both His-107 residues (one from each monomer) appear to be involved in an aromatic stacking interaction with Tyr-146, a residue conserved in all bacterial hypB homologs as either a Tyr or Phe (Fig. 4A). This aromatic residue is just downstream of the Switch II motif that includes the metal ligand Cys-142, so metal affinity could be indirectly influenced by the π-stacking interaction between His-107 and Tyr-146, but additional work will be required to test this possibility.

In the case of zinc binding to hypB, there is no evidence for an analogous change in metal coordination. In contrast to the observation with nickel, the Zn(II) stoichiometry of hypB was not altered in the presence of nucleotide, and zinc did not activate dimerization in the same manner as nickel (30). Solution studies of hypB and ecHypB are consistent with His-107 serving as a zinc ligand in the absence of nucleotide (28, 30). Furthermore, the crystal structure of MjHypB loaded with Zn(II) and a GTP analog revealed that one histidine, His-96, from one monomer acts as a Zn(II) ligand (32). The fact that His-107 coordinates zinc in the presence of nucleotide but not nickel suggests that it may play a role in distinguishing between the two metals.

A comparison of the Ni(II)-hypB and Zn(II)-MjHypB structures revealed that they are globally very similar. Neither protein contains the N-terminal high affinity nickel-binding site or histidine-rich extension present in other hypB homologs (Fig. 4A), so they should be comparable from a functional point of view. The G1, G2, and G3 motifs from both structures are superimposable, and the GDP + P bound hypB correspond to the GTP-bound state of MjHypB. In many G-proteins, the enzyme is viewed as a molecular switch in which the GTP-bound state is considered to be an “on” conformation (65). Some local differences between the hypB and MjHypB metal-bound structures were observed in the manner that the two proteins interact with the guanine base. In the Ni(II)-hypB structure, the regions involved in GTP binding are in a conformation allowing for more solvent accessibility, which may provide an explanation for the less dramatic inhibition of GTP hydrolysis by nickel than when zinc is bound. In addition, subtle shifting of several surface helices could impact protein-protein interactions during metallocenter assembly. How the combination of metal and GTP binding influences the interactions of hypB with partner proteins is a key issue that remains to be defined.

The results presented in this report suggest that Cys-142 serves as a molecular link between the metal-binding site and the GTPase activity. Mutation of Cys-142 to a non-ligating serine resulted in weakened metal binding to hypB under all conditions investigated, suggesting that this residue is always involved in metal coordination regardless of nucleotide-bound state. This result is consistent with the available structural data (32) as well as analysis of the corresponding C198A mutation in ecHypB that disrupted nickel binding without abrogating it altogether (28). Furthermore, removing Cys-142 decouples the GTPase and metal binding activities of hypB. Zn(II) loading of the mutant protein yielded a kcat only 2-fold lower than that of apo-C142S instead of reducing the GTPase activity to barely detectable levels as it did in the WT protein. This result is consistent with the location of Cys-142 in the Switch II motif, a critical region of GTPases responsible for transducing ligand binding events to altered catalysis (57).

Cys-142 is in a unique position because it can link dimer formation and nucleotide switching to metal coordination. In many GTPases, the Switch II region is flexible (66) and often undergoes conformational rearrangement upon GTP hydrolysis (56). This general mechanism is adapted to the individual GTPase. One example is the nitrogenase subunit NiFH, which catalyzes the ATP hydrolysis required for reduction of dinitrogen and is in the same class of NTPases as hypB (54). The crystal structure of NiFH revealed that a conserved Cys residue in the Switch II motif coordinates an Fe₅S₄ cluster (67). Analysis of the structure, which was loaded with ADP and AlF₄⁻ in an effort to mimic the transition state, revealed that the Switch II motif connects binding of the AlF₄⁻ to contacts with another subunit (67). This connection led to the proposal that the nucleotide switch motif couples electron transfer with ATP hydrolysis through intersubunit stabilization (67). By analogy, the hypB Switch II motif could couple metal binding to GTP hydrolysis and protein–protein interactions. In the available crystal structures of hypB, the Switch II motif is located at the homodimer interface, so the nucleotide-mediated conformational changes could influence the structure of the hypB dimer. Alternatively, this switch in hypB could impact the interactions between hypB and other accessory proteins of the hydrogenase biosynthetic pathway.

The interplay between nucleotide binding and metal binding in hypB may be a key aspect of the role of this protein within the hydrogenase biosynthetic pathway. The data presented in this report suggest that the differences between the nickel and zinc complexes of hypB could provide the means for metal ion discrimination, thus ensuring that only the cognate nickel ion is delivered to the hydrogenase precursor protein. Future research will reveal how the activities of the protein are affected by other components that are required to deliver nickel to hydrogenase, whether these properties of hypB are conserved in other homologs, and how they impact the role of this
protein in the biosynthesis of urease, the other nickel enzyme in H. pylori.

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