Metal Binding and Response of *Helicobacter pylori* HypB and *Escherichia coli* YjiA

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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Abstract

The biosynthesis of [NiFe]-hydrogenase and urease in Helicobacter pylori requires several accessory proteins for proper assembly of the nickel-containing active sites. Critical to the maturation of both enzymes in H. pylori is the GTPase HypB. In this work, the metal-binding properties of H. pylori HypB (HpHypB) were investigated and a link between metal binding and the other biochemical properties of HpHypB was established. HpHypB binds stoichiometric nickel or zinc with nanomolar affinities, in partially overlapping sites located between two major GTPase motifs. Upon metal binding, the GTP hydrolysis activity and oligomeric properties of the protein are modulated. Furthermore, the stoichiometry and affinity of the nickel is altered when HpHypB is bound to nucleotide, a change not observed for zinc. Mutagenesis of the metal ligands suggest that a conserved cysteine is responsible for transducing the metal-bound state to altered GTPase activity and a conserved histidine is a required nickel ligand only in the nucleotide-bound state. Together, these results suggest that the metal-binding and GTP hydrolysis properties of HpHypB are intimately linked and may comprise a mechanism through which the [NiFe]-hydrogenase and urease maturation pathways can discriminate between Ni(II) and Zn(II). Characterization of the Escherichia coli GTPase YjiA, a member of the same GTPase family as HpHypB, demonstrated that YjiA can bind Ni(II), Zn(II), or Co(II) at a site in a similar
location as in $Hp$HypB. Metal binding also regulates the GTPase activity and oligomerization of YjiA. This finding suggests that metal-responsive GTPase activity may be a trait of this family of GTPases. Together, this work describes a unique link between the metal-binding and biochemical properties of the G3E GTPases and provides insight into the role of $Hp$HypB in [NiFe]-hydrogenase and urease maturation.
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# Table of Contents

Acknowledgments .......................................................................................................................... iv  
Table of Contents ........................................................................................................................... vi  
List of Tables ................................................................................................................................. ix  
List of Figures ............................................................................................................................... xi  
List of Appendices ....................................................................................................................... xiv

1 Introduction ................................................................................................................................ 1  
  1.1 Nickel in Biological Systems .............................................................................................. 1  
  1.2 The Chemistry of Nickel ..................................................................................................... 1  
  1.3 Nickel Transport ................................................................................................................. 3  
    1.3.1 Nickel Importers ..................................................................................................... 4  
    1.3.2 Nickel Export .......................................................................................................... 6  
  1.4 Nickel Enzymes: Structure and Assembly .......................................................................... 7  
    1.4.1 [NiFe]-Hydrogenase ............................................................................................... 7  
    1.4.2 Urease ................................................................................................................... 13  
    1.4.3 Glyoxalase I .......................................................................................................... 16  
  1.5 Nickel Storage Proteins ..................................................................................................... 16  
  1.6 Nickel-Based Genetic Regulation ..................................................................................... 18  
    1.6.1 NikR ...................................................................................................................... 18  
    1.6.2 RcnR ..................................................................................................................... 21  
    1.6.3 E. coli YqiI ............................................................................................................ 21  
  1.7 Purpose of Study ............................................................................................................... 22  
  1.8 References ......................................................................................................................... 23  

2 The Effects of Metal on the Biochemical Properties of *Helicobacter pylori* HypB, a Maturation Factor of [NiFe]-Hydrogenase and Urease ................................................................. 42 
  2.1 Introduction ......................................................................................................................... 42
Appendix I: Elucidation of the Mechanism of Nickel-Induced DNA Binding by *Escherichia coli* NikR: An Attempt to Engineer a Nickel-Independent NikR ......................................................... 173

AI.1  Introduction………………………………………………………………………………173

AI.2  Materials and Methods………………………………………………………………175

AI.3  Results……………………………………………………………………………………180

AI.4  Discussion………………………………………………………………………………186

AI.5  References………………………………………………………………………………188

Appendix II: DYNAFIT Scripts ................................................................................. 191

AII.1  Competition Between *Hp*HypB and MF2 for Ni(II)…………………………...191

AII.2  *Hp*HypB Ni(II) vs Zn(II) Competition………………………………………………192

AII.3  Competition Between Mg(II)-loaded *Hp*HypB and MF2 for Ni(II)…………193

AII.4  Competition Between Nucleotide-loaded *Hp*HypB and MF2 for Ni(II)……194

AII.5  Competition between Mg(II)- and Nucleotide-loaded *Hp*HypB and MF2 for Zn(II).195
List of Tables

Table 2-1  PCR primers used for cloning and mutagenesis................................. 45
Table 2-2  Stoichiometry of metal binding to WT and mutant \textit{HpHypB}................. 53
Table 2-3  Summary of gel filtration chromatography results of WT and mutant \textit{HpHypB} with added metals................................................................. 57
Table 2-4  Summary of gel filtration chromatography results of WT \textit{HpHypB} with added nucleotides................................................................. 58
Table 2-5  Kinetics of GTP hydrolysis by \textit{HpHypB}............................................. 60
Table 3-1  PCR primers used for mutagenesis...................................................... 74
Table 3-2  Calculated and observed molecular masses (MM) for WT and mutant \textit{HpHypB} as determined by ESI-MS......................................................... 76
Table 3-3  Data collection and refinement statistics for data sets used to solve the \textit{HpHypB} structure and to determine the presence of nickel at the binding site................................................................. 86
Table 3-4  Kinetics of GTP hydrolysis by WT, H107A, and C142S \textit{HpHypB}........... 88
Table 3-5  Apparent Ni(II) \textit{K}\textsubscript{d} for WT and mutant \textit{HpHypB} with or without nucleotide 92
Table 3-6  Melting temperatures of WT and C142S \textit{HpHypB} determined by circular dichroism spectroscopy................................................................. 93
Table 3-7  Affinity of nucleotide binding to WT, H107A, and C142S \textit{HpHypB}........... 95
Table 3-8  Stoichiometry of metal binding to WT \textit{HpHypB}..................................... 98
Table 4-1  PCR primers used for cloning and mutagenesis................................... 124
Table 4-2  Calculated and observed molecular masses (MM) for WT \textit{YjiA} and mutants, as determined by ESI-MS......................................................... 126
Table 4-3  Crystallographic data collection and refinement statistics..................... 134
Table 4-4  Stoichiometry of metal binding to WT and mutant \textit{YjiA}....................... 135
Table 4-5  Summary of gel filtration chromatography results of WT and mutant \textit{YjiA} with added metals and nucleotide......................................................... 143
Table 4-6  Kinetics of GTP hydrolysis by WT and E37A,C66A,C67A \textit{YjiA}........... 144
Table AI-1  Electrospray ionization mass spectrometry results for S69C NikR oxidation methods
# List of Figures

| Figure 1-1 | Summary of protein systems involved in *Escherichia coli* and *Helicobacter pylori* nickel homeostasis | 2 |
| Figure 1-2 | Structure of the *Escherichia coli* NikA periplasmic-binding protein | 5 |
| Figure 1-3 | The active sites of nickel-containing enzymes found in *Escherichia coli* and *Helicobacter pylori* and the reactions catalyzed | 8 |
| Figure 1-4 | NMR structure of *Helicobacter pylori* HypA | 10 |
| Figure 1-5 | Structure of *Methanocaldococcus jannaschii* HypB | 11 |
| Figure 1-6 | NMR Structure of *Helicobacter pylori* SlyD | 12 |
| Figure 1-7 | Structure of *Helicobacter pylori* urease accessory protein UreE | 15 |
| Figure 1-8 | Structure of the nickel-responsive transcription factor *Escherichia coli* NikR in complex with DNA | 19 |
| Figure 2-1 | Amino acid alignment of *Hp*HypB with homologs | 44 |
| Figure 2-2 | Nickel binding to *Hp*HypB | 52 |
| Figure 2-3 | The affinity of *Hp*HypB for Ni(II) | 53 |
| Figure 2-4 | The affinity of WT and C106A, H107A *Hp*HypB for Zn(II) | 55 |
| Figure 2-5 | The effect of metal on the quaternary structure of *Hp*HypB | 56 |
| Figure 2-6 | The GTPase activity of *Hp*HypB | 60 |
| Figure 2-7 | Homology model of *Hp*HypB | 62 |
| Figure 3-1 | Timecourse of GTP hydrolysis by apo-WT *Hp*HypB | 84 |
| Figure 3-2 | GTPase activity of *Hp*HypB | 89 |
| Figure 3-3 | Dependence of $k_{cat}$ on [KCl] | 90 |
| Figure 3-4 | Effect of metal and nucleotide on the quaternary structure of WT *Hp*HypB | 90 |
| Figure 3-5 | Affinity of *Hp*HypB for Ni(II) and Zn(II) in potassium-containing buffer | 91 |
| Figure 3-6 | Thermal denaturation curves of WT *Hp*HypB monitored by circular dichroism spectroscopy | 93 |
| Figure 3-7 | Circular dichroism (CD) spectra of WT, H107A, and C142S *Hp*HypB | 94 |
Figure 3-8  Nucleotide binding to WT and mutant *HpHypB*..............................96
Figure 3-9  Nickel binding to WT *HpHypB* in the presence of GDP and GDPNP...........98
Figure 3-10 Nickel affinity of WT *HpHypB* in the presence of nucleotide.....................99
Figure 3-11 Zinc affinity of WT *HpHypB* in the presence of nucleotide.....................100
Figure 3-12 The crystal structure of Ni(II)- and GDP + P_i-bound *HpHypB*...................103
Figure 3-13 Overlay of *HpHypB* and *MjHypB*..............................................104
Figure 3-14 Metal binding to H107A and C142S *HpHypB*...................................106
Figure 3-15 Effect of metal and nucleotide on the quaternary structure of H107A and C142S *HpHypB*.................................................................107
Figure 3-16 GTPase activity of H107A *HpHypB*.................................................108
Figure 3-17 Amino acid alignment of *HpHypB* with homologs..............................112
Figure 3-18 Proposed model of Ni(II) vs. Zn(II) discrimination by *HpHypB*.............115
Figure 4-1  Cobalt and nickel binding to WT YjiA..............................................136
Figure 4-2  Zinc binding to YjiA.................................................................137
Figure 4-3  Electrospray ionization mass spectrum of WT YjiA.................................138
Figure 4-4  Structure of WT YjiA and location of the metal-binding site in the primary structure of the GTPase domain.................................................139
Figure 4-5  Circular dichroism (CD) spectrum of WT YjiA and two YjiA triple mutants141
Figure 4-6  Effect of metal on the quaternary structure of YjiA.................................142
Figure 4-7  Crystal structure of Zn(II)-bound WT YjiA........................................146
Figure 4-8  Comparison of crystal lattices of apo-WT YjiA and Zn(II)-soaked WT YjiA.................................................................147
Figure 4-9  The Walker A motif (P-loop) adopts multiple conformations in YjiA..........148
Figure 4-10 E37 or E39 of YjiA could coordinate nucleotide-associated Mg(II)..............149
Figure 4-11 Crystal structure of Zn(II)-soaked E37A,C66A,C67A YjiA..........................151
Figure 4-12 Comparison of the metal-binding sites of YjiA and HypB..........................154
Figure A1-1 Design rational for the S69C NikR mutant...........................................174
**Figure A1-2** Oxidation of S69C NikR using 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB). 181

**Figure A1-3** Nickel titration and difference spectrum of S69C NikR……………………. 184

**Figure A1-4** Circular dichroism (CD) spectra of WT and S69C NikR……………………. 185

**Figure A1-5** Binding of nickel-loaded WT and S69C NikR to the *nik* promoter……….. 186
List of Appendices

Appendix I  Elucidation of the Mechanism of Nickel-Induced DNA Binding by
*Escherichia coli* NikR: An Attempt to Engineer a Nickel-Independent NikR.................................................................172

Appendix II  DYNAFIT Scripts............................................................................................................ 191
1 Introduction

1.1 Nickel in Biological Systems

Nickel is an essential metal required by many eubacteria, archaebacteria, fungi, and plants for various biological processes including the global carbon cycle, energy metabolism, and bacterial pathogenesis. To date, several enzymes requiring nickel as a cofactor have been discovered, including [NiFe]-hydrogenase, urease, Ni-containing glyoxylase, carbon monoxide dehydrogenase/acetyl-coA synthase, Ni-superoxide dismutase, methyl-coenzyme M reductase, acireductone dioxygenase, and the more recently discovered methyleneurease from the *Burkholderia* species, *B. subtilis* glycerol-1-phosphate dehydrogenase AraM, and the monocupin dioxygenase quercetinase from *Streptomyces* sp. FLA. Despite being critical for the survival of many organisms, excess nickel is toxic for a variety of reasons, including the replacement of the cognate metals in iron or zinc enzymes, nickel-induced allosteric inhibition of enzymes, and the indirect activation of oxidative stress. In order to meet the nutritional requirements and avoid toxic buildup of the metal, organisms have evolved complex nickel homeostasis systems that combine nickel uptake and export with genetic regulation, cytosolic nickel storage, and directed insertion of the metal into the enzyme active sites. A significant body of knowledge about nickel in prokaryotic systems is known and has been thoroughly reviewed. This introduction will be limited to the nickel homeostasis pathways in *E. coli* and *H. pylori* (Figure 1-1). Relevant protein homologs from other species will also be discussed if they contribute to the understanding of the *H. pylori* and *E. coli* systems.

1.2 The Chemistry of Nickel

Nickel is a first row d transition metal found primarily in the +2 oxidation state, but it also occurs in formal 0, +1 and +3 oxidation states in biological systems. Four-coordinate Ni(II) complexes are typically square planar, with tetrahedral geometries less common, whereas trigonal-bipyramidal and square pyramidal geometries are frequently associated with five-

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coordinate Ni(II) and six-coordinate Ni(II) is octahedral. The square planar geometry, which is only shared with Cu(II) in biological systems, is a result of the d^8 electron configuration of Ni(II), wherein the planar tetra-ligand coordination causes the d_x^2-y^2 orbital to be uniquely high in energy and thus left vacant. It should be noted that Zn(II) is unlikely to form square planar coordination geometries due to its closed d^{10} electron configuration, a fact important in metal selectivity. Moving from left to right across the first row, the transition metals become
increasingly softer. Nickel is situated within the borderline region of this trend, preferring nitrogen ligands, but is also frequently bound to the softer sulfur and harder oxygen ligands.\(^{32}\) Moreover, given its position in the Irving-William series of small molecule complex stabilities,\(^{33}\) Mn(II) < Fe(II) < Co(II) < Ni(II) < Cu(II) > Zn(II), nickel tends to form relatively tight complexes.

In many cases, the use of nickel in enzymatic reactions correlates with ancient processes that arose at the onset of life near volcanic vents where iron, nickel, H\(_2\), CO\(_2\), and CO were abundant.\(^{29,32}\) During this period of Earth’s history, soluble nickel was more accessible than zinc and copper, two metals frequently found in many modern bacterial metallomes.\(^{32}\) Around 2.4 billion years ago, a decrease in atmospheric methane and an increase in oxygen production lead to the Great Oxidation Event,\(^{34}\) which radically altered the relative availability of soluble metals and ultimately led to oxygen-dependent multi-cellular life.\(^{35}\) The decrease in methane production has been attributed to the starvation of methane-producing bacteria for nickel,\(^{34}\) which may have been due to the cooling of the Earth’s mantle resulting in decreased eruption of nickel-rich rocks and an estimated 50% decrease in oceanic nickel concentrations.\(^{34}\) The nickel-dependent methane production in these bacteria is due to methyl-coenzyme M reductase, a nickel-containing enzyme that is involved in the final step of the methane biosynthetic pathway within methanogenic archaea and that is responsible for all biologically produced methane.\(^{1,2}\) Despite this decrease in nickel availability, there remain a multitude of systems for which Ni(II) is the ideal metal, many of which will be discussed throughout this chapter.

1.3 Nickel Transport

To prevent a buildup of toxic nickel, nickel transport across the cell membrane must balance uptake and export with the nutritional requirements of the organism. To drive nickel transport, there are a variety of known nickel proteins that harness cellular energy sources such as ATP or potentiometric gradients. Some nickel may also enter the cell in a non-specific manner via other uptake systems such as the inner membrane CorA that mediates the transport of divalent metal ions such as Mg(II).\(^{36-38}\) Although Ni(II) can pass through this system, the \(K_m\) is \(\sim 300 \mu\text{M}\), calling into question the physiological relevance of this uptake mechanism,\(^{39,40}\) as environmental nickel concentrations tend to be in the nM range, as is the case of sea water\(^{41}\) and human blood
Of the various specific nickel transporters discussed below, only a subset may be found in a particular organism.

### 1.3.1 Nickel Importers

#### 1.3.1.1 ABC Ni(II) Transporters

The ATP-binding cassette (ABC) transporters are the most widely used pathways for moving Ni(II) across the cytoplasmic membrane, represented by the NikABCDE protein in \textit{E. coli}.\textsuperscript{43} These multicomponent systems generally consist of integral membrane channels and associated ATP-hydrolyzing proteins to provide energy for transport, with a soluble periplasmic Ni(II)-binding protein as an additional component of NikABCDE.\textsuperscript{44} \textit{Escherichia coli} NikABCDE is the best characterized Ni(II) transporter to date, serving as the main source of nickel for several [NiFe]-hydrogenases in this organism.\textsuperscript{45-49} In this system, NikA is the periplasmic binding protein (PBP), which presumably picks up nickel in the periplasm and delivers it to NikB and NikC, which form the heterodimeric transmembrane core and are coupled to the ATP-hydrolyzing NikD and NikE proteins.\textsuperscript{45}

\textit{E. coli} NikA is a mixed \(\alpha/\beta\) PBP capable of binding a single nickel ion per monomer in a cleft between two globular domains (Figure 1-2).\textsuperscript{50,51} Originally, crystallographic and spectroscopic evidence suggested that nickel is bound as a complex with a small organic molecule contributing several carboxylate ligands, modeled as butane-1,2,4-tricarboxylate (BTC).\textsuperscript{51-53} However, assignment of the molecule as BTC is suspect, given that no biosynthetic pathway for this molecule is known in \textit{E. coli}. A recent report suggests that the substrate for the transporter is instead a Ni(L-His)\(_2\) complex\textsuperscript{54} and a crystal structure with this complex bound to NikA has been determined (Figure 1-2),\textsuperscript{55} but definitive assignment is still pending. An essential histidine ligand (His416) also coordinates the Ni(II).\textsuperscript{52,56} Given the clear parallels with bacterial siderophores used to assimilate Fe(III),\textsuperscript{57} it is reasonable that \textit{E. coli} and other bacteria utilize a small-molecule chelator to compete for low abundance nickel in the environment. It should be noted that the NikABCDE transporter is phylogenetically more closely related to oligopeptide transporters than to other metal transporters,\textsuperscript{58} supporting the hypothesis that NikABCDE transports a nickel complex rather than a “naked” nickel ion.
1.3.1.2 Secondary Nickel Transporters

The secondary nickel transporters are high-affinity permeases that allow passive transport of nickel across the cytoplasmic membrane. As opposed to the multi-component ABC systems, these transporters consist of a single polypeptide chain. The nickel/cobalt transporter (NiCoT) family is widely distributed, spanning all three domains of life, and is characterized by an eight transmembrane-domain (TMD) architecture and a conserved HX₄DH motif located in the second transmembrane segment. Central to the transport of metals ions are the His and Asp residues in the conserved HX₄DH motif. NiCoTs work efficiently when their substrates are present in the low nM range. The NiCoT family member in *H. pylori*, NixA, facilitates high-affinity Ni(II) transport ($K_T = 11.3$ nM), and is inhibited by Co(II), Cu(II), and Zn(II). The ability of the Ni(II)-permeases to facilitate high-affinity and specific nickel uptake is not well understood.
1.3.1.3 Outer Membrane Transporters

In gram-negative bacteria, such as *E. coli* and *H. pylori*, cellular nutrients must pass through an outer membrane before reaching the cytoplasmic membrane. One mechanism for Ni(II) transport across the outer membrane is through trimeric β-barrel proteins known as porins, which allow for the nonselective passive diffusion of metal ions.\(^{64}\) However, Ni(II) may cross the outer membrane through other mechanisms as well. In the case of complexes, such as the putative small molecule Ni(II) complex bound by NikA, a TonB-dependent transporter (TBDT) system is needed.\(^ {65}\) TonB spans the periplasm and couples the transport of larger species across the outer membrane to inner membrane protein complexes (ExbB and ExbD), which access the local proton gradient.\(^ {65,66}\) Nickel-transporting TBDTs have been characterized in *Helicobacter* species, where a TonB ortholog contributes to Ni(II) acquisition\(^ {67}\) and is proposed to energize NikH,\(^ {67,68}\) FecA3 and FrpB4,\(^ {69-71}\) all of which are outer membrane Ni(II) transporters. In particular, the TBDT-FrpB4 system is dedicated to nickel accumulation at low pH, thereby allowing the pathogenic *H. pylori* to colonize the acidic human stomach by activation of apo-urease with an influx of Ni(II).\(^ {71}\) Details of the transport mechanisms of these proteins and the identity of their substrates are unknown.

1.3.2 Nickel Export

One mechanism that organisms use to avoid nickel toxicity is nickel efflux. To date, nickel efflux proteins can be broadly classified into three major categories: RcnA, Major Facilitator Protein Superfamily (MFS), and the Resistance-Nodulation-Cell Division (RND)-driven exporters, of which the RcnA and RND-driven exporters have been identified in *E. coli* and *H. pylori*, respectively.

Resistance to cobalt and nickel protein A (RcnA) is the only nickel efflux protein identified in *E. coli*.\(^ {72}\) The *rcnA* gene product includes a His-rich loop consisting of 17 His, 3 Asp and 3 Glu in a 26-residue stretch.\(^ {72}\) Upon inactivation of the *rcnA* gene *E. coli* become more sensitive to Ni(II) and Co(II) and accumulate more Ni(II).\(^ {72,73}\) The proposed topology of RcnA includes 6 TMDs and does not bear significant homology with other transporters.\(^ {72}\) The *rcnB* gene located directly downstream of *rcnA* is co-expressed with *rcnA* and encodes a soluble periplasmic protein.\(^ {74}\) Deletion of *rcnB* results in increased Ni(II) and Co(II) resistance and a decrease in the overall
cellular levels of the two metals, suggesting that this protein may regulate the efflux activity of RcnA through an unknown mechanism.\textsuperscript{74}

The RND-driven exporters are found in all major domains of life and are responsible for transporting a wide variety of substrates including heavy metals, hydrophobic compounds, and proteins.\textsuperscript{75} RND proteins consist of 12 transmembrane helices with the metal-transporting members containing the consensus sequence DFGX\textsubscript{3}DGAX\textsubscript{3}VEN in TMD IV.\textsuperscript{75} It is believed that a cycle of protonation/deprotonation of the acidic residues in this sequence is responsible for driving the translocation of the substrate out of the cell.\textsuperscript{75} Several major Ni(II)-transporting variants of the RND-driven exporters are known, such as CznABC in \textit{H. pylori},\textsuperscript{76} none of which are specific for just Ni(II).

1.4 Nickel Enzymes: Structure and Assembly

1.4.1 [NiFe]-Hydrogenase

Hydrogenase enzymes catalyze the reversible oxidation of hydrogen gas to protons and electrons (Figure 1-3), a process central to microbial energy metabolism.\textsuperscript{3,77} [NiFe]-hydrogenases are found mainly in archaea and bacteria\textsuperscript{78,79} and, in a few notable cases such as pathogenic \textit{H. pylori}, are critical for colonization.\textsuperscript{3} [NiFe]-hydrogenases are heterodimers with the [NiFe] active site in a large subunit (LS) and a series of iron-sulphur clusters responsible for electron transfer in the small subunit (SS).\textsuperscript{80} In the active site, nickel is coordinated to four Cys thiolates, two of which bridge to the iron center which also has one carbon monoxide and two cyanide ligands (Figure 1-3).\textsuperscript{80}

Although phylogenetically distinct, [NiFe]- and [FeFe]-hydrogenases appear to be a case of evolutionary convergence as the two enzymes have remarkably similar active sites and both catalyze the same chemical reaction.\textsuperscript{78,81} There is no consensus regarding the role of nickel in [NiFe]-hydrogenase, making the reason for its selection over other metals a source of speculation. It has been proposed that the presence of nickel in [NiFe]-hydrogenase is due to relatively high nickel availability during Earth’s early history.\textsuperscript{32} The presence of CO and CN
ligands, as well as putative inorganic molecules bridging the two metals, draw parallels to those naturally occurring inorganic compounds found in primordial Earth near volcanic vents.28,82

Due to the complexity of the metal site, the assembly of [NiFe]-hydrogenases requires an array of accessory proteins, most encoded by the hyp (hydrogenase pleiotropic) operon.83 This process has been best characterized in E. coli, where at least 3 hydrogenase isoenzymes are expressed.24 Following ribosomal translation of the gene encoding the LS, biosynthesis of the cluster is believed to occur in two stages, with the iron inserted first, followed by nickel.84 The iron is incorporated into the LS (encoded by hycE for hydrogenase 3) by HypCDEF, which are also thought to be responsible for the synthesis and incorporation for at least one of the diatomic ligands.84-87 HypC remains associated with HycE until the metal center is complete,88,89 possibly holding the LS in a conformation appropriate for nickel insertion, which is achieved by HypA, HypB and SlyD and is discussed in detail below. HypC then dissociates from the LS and proteolytic cleavage of the C-terminal tail by an isoenzyme-specific protease occurs.85,90 A conformational change then yields the mature LS that can associate with the SS, prepared by a parallel biosynthetic pathway.79,85

Figure 1-3. The active sites of nickel-containing enzymes found in Escherichia coli and Helicobacter pylori and the reactions catalyzed. Structures shown: [NiFe] hydrogenase, Desulfovibrio gigas (PDB 1FRV), oxidized inactive form where X is a small molecule (O in the D. gigas crystal structure); Glyoxalase I, Escherichia coli (PDB 1F9Z); Urease, Klebsiella aerogenes (PDB 1FWE).
1.4.1.1 HypA

HypA is believed to serve as the scaffold for assembly of the nickel insertion proteins with the [NiFe]-hydrogenase large subunit. HypA can interact with HypB and HycE, and there is evidence that HypA forms a complex with HypB and SlyD in the absence of HycE, suggesting that the nickel-insertion complex can pre-assemble prior to association with the hydrogenase precursor protein.

HypA consists of 2 metal-binding domains. Both HypA and its homolog HybF, which substitutes for HypA in the biosynthesis of E. coli hydrogenases 1 and 2, bind stoichiometric nickel with micromolar affinity. This N-terminal Ni(II) site includes the conserved His2, which is essential for hydrogenase activity, but the rest of the site is under debate. Spectroscopic analysis of H. pylori HypA with extra N-terminal residues supports a square planar coordination including the terminal amine and the backbone nitrogens of two nearby residues, whereas XAS of unmodified protein suggested that the site may instead be 5-6 coordinate, with a coordination sphere composed of 3-4 (N/O) and 1-2 His ligands.

HypA and HybF also bind stoichiometric zinc (E. coli HypA K_D-Zn(II) = 0.9 nM) in a site composed of cysteines from two conserved CXXC motifs, similar to the coordination sphere found in structural zinc sites (Figure 1-4). In H. pylori HypA (HpHypA), this Zn(II) site is modulated by both nickel loading and pH changes. At the diminished pH of 6.3, consistent with the cytoplasm of bacteria exposed to acid shock, the zinc site undergoes ligand substitution to convert the Zn(II)Cys4 site to a Zn(II)HisCys3 site. If nickel is also present at these lower pH values, further rearrangement of the Zn(II) site occurs to yield a Zn(II)His2Cys2 coordination. Furthermore, the decrease in pH also reduces the nickel stoichiometry from one ion per monomer to one ion per dimer. In H. pylori, HypA also contributes to urease biosynthesis and these pH- and Ni(II)-dependent changes to the Zn(II) coordination sphere of HypA are proposed to direct Ni(II) to the different enzyme assembly pathways by favoring or disfavoring interaction with HypB or the urease accessory protein UreE.
1.4.1.2 HypB

HypB is a GTPase essential for the synthesis of hydrogenase and contributes to nickel delivery in cooperation with HypA. All HypB homologs possess a low-affinity metal-binding site located between two of the conical GTPase motifs in the C-terminal part of the protein, which is required for hydrogenase biosynthesis in \textit{E. coli}. This site binds one nickel ion with a $K_D = 12 \mu M$ or $K_D = 150 \text{nM}$ for the \textit{E. coli} (EcHypB) and \textit{H. pylori} (HpHypB) homologs, respectively, with the coordination environment involving a conserved CH motif (Chapter 2). However, Zn(II) binds 10-100 times more tightly, prompting speculation regarding the identity of the physiologically relevant metal. In the crystal structure of HypB from \textit{Methanocaldococcus jannaschii}, two Zn(II) are bound at an asymmetric site at the dimer interface with the first ion bound by water and three Cys residues, one of which bridges to a second Zn(II) bound by another Cys, one His, and one water (Figure 1-5). A recent crystal structure of the HypB homolog from \textit{Archeoglobus fulgidus} lacks bound metal, but in that report, the authors proposed that the metal site could feature a single nickel ion bound between two monomers in a tetrathiolate coordination environment, in a location similar to the ZnA in the \textit{MjHypB} structure (Figure 1-5). However, the relevance of these coordination environments in

\begin{figure}
\centering
\includegraphics[width=\textwidth]{hypB.png}
\caption{NMR structure of Helicobacter pylori HypA (PDB: 2KDX). The overall structure of \textit{HpHypA} is a mixed $\alpha/\beta$ structure consists of two domains: a zinc domain and a nickel domain. Due to the unstructured nature of the nickel-binding site, it was not resolved in the structure. The structural zinc site is shown here with the metal coordinated to four Cys residues from the two CXXC motifs. The color scheme is zinc, grey sphere; and sulfur, yellow. The images were generated using PyMol.}
\end{figure}
vivo is not clear as a mutant EcHypB lacking the ability to dimerize can still bind metal in the low-affinity site and function in hydrogenase biosynthesis.\textsuperscript{114}

Isolated HypB displays low levels of GTPase activity that is believed to serve a regulatory role in hydrogenase maturation,\textsuperscript{105} and disruption of this activity abolishes hydrogenase production due to lack of nickel incorporation into the large subunit.\textsuperscript{105} In HpHypB, Zn(II) binding to the low-affinity site abolishes GTPase activity whereas nickel had little effect on catalysis (Chapter 2).\textsuperscript{112} In contrast, both metals impair GTPase activity in EcHypB, with Zn(II) imparting the more inhibitory effect.\textsuperscript{114}

In addition to the invariant metal site in the GTPase domain, EcHypB possesses a high-affinity nickel-binding sequence CXXCGC at the N-terminus of the protein.\textsuperscript{111} The Ni(II) binds in a square planar site with three Cys and the N-terminal amine serving as ligands with a $K_D \approx 0.1$ pM.\textsuperscript{110,111,115,116} Although not conserved in all HypB homologs, this site is critical for hydrogenase maturation in \textit{E. coli}.\textsuperscript{110}
1.4.1.3 SlyD

SlyD was isolated with hydrogenase biosynthetic factors in pull down experiments in *E. coli* and *H. pylori*. Deletion of *E. coli* slyD results in reduced hydrogenase activity, a phenotype suppressed by the addition of excess nickel in the growth media. The available structures of SlyD reveal a multi-domain protein consisting of a peptidyl-prolyl isomerase (PPIase) domain and a chaperone domain as well as an unstructured C-terminal metal-binding region (Figure 1-6). The C-terminal sequence is rich in potential metal-binding residues, including 15 histidines, 6 cysteine, and a dozen carboxylate amino acids, and is required for hydrogenase maturation, unlike the PPIase activity. SlyD is proposed to contribute to several aspects of hydrogenase maturation. SlyD may function as a general chaperone given that it interacts with HycE prior to iron and nickel insertion. In addition, SlyD can act as a source of nickel for HypB and modulate both the high-affinity nickel-binding site of HypB and GTP hydrolysis, revealing a link between nickel release from HypB and the GTPase activity of the protein.

In addition to its role in hydrogenase maturation, SlyD acts as a nickel storage protein in *E. coli*, as deletion of slyD results in lower nickel accumulation in vivo, and it also impacts the activity of the nickel responsive transcription factor NikR. The full-length protein can bind up

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**Figure 1-6. NMR structure of *Helicobacter pylori* SlyD.**
The structure of SlyD consists of multiple domains: the IF domain (at bottom) and the PPIase domain (top domain). The highly unstructured metal-binding domain was not resolved in this structure. It is within this domain that SlyD can bind multiple metal ions. The image was generated in PyMol.
to seven nickel ions in a non-cooperative manner with an overall apparent $K_D < 0.1 \text{ nM}$.$^{128,129}$

The Ni(II) is bound in several different geometries with His and Cys residues from the C-terminal tail serving as ligands.$^{129,130}$ The protein also binds a variety of other metals with a trend similar to the Irving-Williams series, but only Ni(II) binding appears to be relevant in vivo.$^{128}$

Given that the metal-binding domains (MBDs) of SlyD homologs vary considerably, it is unclear how conserved these roles of SlyD are.

### 1.4.2 Urease

Urease, the only nickel enzyme found in eukaryotes,$^5$ catalyzes urea hydrolysis to yield ammonia and carbamate, which further decomposes into a second molecule of ammonia as well as carbon dioxide (Figure 1-3). This reaction is a key component of the global nitrogen cycle and is critical for many pathogenic bacteria, which utilize the ammonia as a means to buffer environmental pH changes.$^5,7,131$ The catalytic center of urease contains two nickel ions: one is bound in a square pyramidal environment to two histidines and one water molecule, as well as a carbamylated lysine and a second water serving as bridging ligands. The pseudo-octahedral coordination sphere of the second nickel is completed by two histidines, an aspartic acid, and a water molecule (Figure 1-3).$^5,6,132$ The ligands are pre-organized in the apo-enzyme,$^{133}$ likely contributing to the high affinity and specificity of the enzyme for nickel ions.$^{131}$ The choice of Ni(II) for urea hydrolysis is not well understood, but may be related to the high affinity of the urea-Ni(II) complex and/or the stability of the Ni(II)-bound active site as compared to similarly coordinated dinuclear Zn(II) centers.$^29$ Furthermore, the recently discovered iron-containing urease applies the same ligand set as nickel-urease, but is less efficient, suggesting that the chemistry of nickel plays a vital role in catalysis.$^{134}$

Much like [NiFe]-hydrogenase, the assembly of the urease active site requires several accessory proteins, UreDEFG.$^6,26,135$ Following translation, apo-urease may be held in an open conformation by GroEL/GroES in order to allow the accessory proteins to carbamylate the active site Lys and to insert the metals.$^{136,137}$ The main function of the accessory proteins appears to be to speed up a very slow process, as apo-urease can be partially activated in vitro in the absence of UreDEFG if supplemented with nickel and a source of CO$_2$.$^{138}$ The functional unit responsible for urease activation is UreDFG.$^{139-142}$ In this complex UreG is a GTPase,$^{140,143,144}$ UreD (called UreH in _H. pylori_) interacts directly with urease$^{145-147}$ and UreF modulates a conformational
change around the urease active site. UreG is capable of binding nickel but biochemical evidence suggests that Zn(II) may be the biologically relevant metal. The proposed Zn(II)-binding site is in the same location relative to the GTPase motifs as the low-affinity site in homologous HypB and likely to adopt a trigonal bipyramidal geometry with two Cys and two His ligands, with the fifth site occupied by a solvent molecule. Metal binding enhances the transition from a partially disordered to structured state in some UreG proteins, the significance of which is currently not understood. Furthermore, Zn(II) stabilizes the interaction between H. pylori UreG and a fourth accessory protein, UreE. The presence of UreE, which is believed to be the source of nickel for urease, decreases the GTP concentration required for urease activation by UreDFG.

1.4.2.1 UreE

Whether or not UreE is critical for urease maturation depends on the organism. In the case of Klebsiella aerogenes deletion of ureE results in a urease-deficient phenotype that can be complemented by nickel, suggesting a nickel metallochaperone function. In contrast, in several Helicobacter species ureE mutations are not complemented by nickel. The crystal structures of Bacillus pasteurii, H. pylori, and truncated K. aerogenes UreE feature a symmetrical homodimer with a metal-binding site at the dimerization interface involving a conserved histidine residue (Figure 1-7). In H. pylori UreE (HpUreE), this site has a $K_D = 0.15 \mu M$ for nickel. Although the crystal structures of HpUreE and B. pasteurii UreE (BpUreE) revealed tetramers, this oligomeric form may not be relevant in vivo. During urease maturation, the position of one UreE dimer in these structures may be replaced by an UreG dimer to form the UreE-UreG complex discussed earlier.

In some organisms, UreE features additional metal-binding capabilities beyond the conserved site at the dimer interface. For example, K. aerogenes UreE (KaUreE) possesses a His-rich C-terminus and can bind 6 nickel ions per dimer in pseudo-octahedral sites composed of O/N donors including multiple histidines with an average apparent $K_D = 9.6 \mu M$. Co(II), Zn(II), and Cu(II) can compete with Ni(II) binding, but they have distinct coordination environments, suggesting that nickel may have a selective allosteric effect on KaUreE. Upon deletion of the His-rich C-terminus, the protein binds two Ni(II) per dimer and this truncated protein is still able to function in urease maturation, so the His-rich motif might instead
contribute to metal sequestration. It is proposed that Ni(II) ions are bound to the periphery of UreE in the resting state and only upon nickel delivery to urease does the conserved histidine at the dimer interface become involved.

1.4.2.2 Connection to [NiFe]-Hydrogenase Maturation

In *H. pylori*, in addition to impacting hydrogenase maturation, *hypA* and *hypB* mutations produce a urease deficiency that is nearly fully complemented by nickel supplementation. This double duty by *HpHypA* and *HpHypB* may be common to the *Helicobacter* species, as it has also been noted in *H. hepaticus*. An interaction between *HpHypA* and *HpUreE* is critical for urease maturation, although no direct nickel transfer from *HpHypA* to *HpUreE* was detected. *HpHypA* competes with *HpUreG* for *HpUreE* binding, possibly serving as a mechanism for Ni(II) delivery to either the urease or [NiFe]-hydrogenase bioassembly pathway. Furthermore, introduction of an additional chromosomal copy of *hpUreE* partially complements mutations in *hypA* and *hypB* and engineered versions of *HpUreE* with His-rich C-terminal sequences yield the greatest urease activity in the *hypA* and *hypB* mutants, demonstrating that there is a correlation between *H. pylori* urease activity and the nickel sequestering ability of UreE. These
experiments suggest that even though wild type HpUreE lacks the His-rich C-terminus found in homologous proteins, *H. pylori* has an alternative way of directing nickel to urease by recruiting the hydrogenase proteins HypA and HypB.\textsuperscript{160}

SlyD also plays an important role in urease maturation in *H. pylori*, which overlaps with that of HypA.\textsuperscript{167} In contrast to *EcSlyD* discussed earlier, the *H. pylori* homolog (*HpSlyD*) binds 2 Ni(II) per monomer with an apparent $K_D = 2.74 \, \mu M$.\textsuperscript{119} The significance of this lower Ni(II)-binding ability is unknown and further characterization of the function of *HpSlyD* in urease maturation is needed.

### 1.4.3 Glyoxalase I

The glyoxalase system, composed of glyoxalase I (GlxI) and glyoxalase II (GlxII), catalyzes the conversion of toxic methylglyoxal to lactate.\textsuperscript{10} Methylglyoxal reacts with glutathione to produce the hemithioacetal substrate of GlxI, which then catalyzes an isomerization reaction to yield S-D-lactoylglutathione, the substrate for GlxII (Figure 1-3).\textsuperscript{10,17} GlxI in yeast and humans is a zinc-dependent enzyme, but the *E. coli* homolog is inactive with Zn(II) and maximally activated with Ni(II).\textsuperscript{168} The active site consists of Ni(II) coordinated in an octahedral geometry by two His, two Glu, and two water molecules (Figure 1-3).\textsuperscript{169,170} This coordination geometry is in contrast to the Zn(II)-bound *E. coli* GlxI which utilizes the same protein ligands but binds the metal in a trigonal bipyramidal geometry with only one water molecule ligand.\textsuperscript{169-171} The enzyme is also activated by other divalent metals, such as Co(II) and Cd(II),\textsuperscript{168,172} which bind in an octahedral geometry, suggesting that this type of geometry at the active site is critical for enzymatic activity.\textsuperscript{10} GlxI binds Zn(II) more tightly than Ni(II),\textsuperscript{172} suggesting that a mechanism for nickel delivery exists, but no information regarding such a system is known.

### 1.5 Nickel Storage Proteins

Nickel sequestration can be achieved by proteins with other functions, as in the case of the His-rich domains of some HypB and UreE homologs and the MBD of *EcSlyD*. Additional proteins that contribute to nickel storage are found in bacteria if the aforementioned domains are missing or the bacteria have a greater need of nickel. For example, in order to maintain sufficient levels of urease and [NiFe]-hydrogenase activities, *H. pylori* accumulates significantly more nickel than *E. coli*,\textsuperscript{173} in part due to the presence of the unique TonB-dependent outer membrane nickel
uptake system and the high-affinity nickel transporter NixA. While the majority of this metal is incorporated into the urease and [NiFe]-hydrogenase enzymes, additional nickel can be stored in the HspA, Hpn and Hpn-like nickel sequestering proteins for times of nickel starvation, to aid in host colonization, and/or to deal with short-term overflow.

HspA is a unique homolog of the GroES family of heat-shock proteins, with an extra C-terminal domain rich in potential metal-binding residues (8 His and 4 Cys in a 27-residue domain). The protein is a heptamer in solution and binds two nickel ions per monomer with an apparent $K_D = 1-2 \mu M$, in a coordination environment consisting of nitrogen and thiolate ligands. One nickel is bound in a square planar Cys$_2$His$_2$ site, with the second site formed by a HX$_4$DH motif, analogous to NiCoTs.

The two-domain structure of HspA suggests that the protein contributes to both classical GroES chaperonin activity as well as nickel-carrier functions. The C-terminal MBD is involved in protecting the cell from nickel toxicity. Heterologous expression of H. pylori urease in E. coli suggested a requirement for HspA in urease maturation, but subsequent experiments in H. pylori did not yield the same results. Instead, HspA mutants in H. pylori impacted hydrogenase maturation, suggesting that HspA substitutes for the lack of the poly-His region in HpHypB or the extended MBD in HpSlyD.

Two other nickel-sequestering proteins from the Helicobacter species are the Hpn and Hpn-like proteins. Hpn contains 28 histidines out of 60 residues and binds 5 Ni(II) per monomer with an apparent $K_D = 7.1 \mu M$. Hpn-like contains 18 His and 30 Gln in a short 72 amino acid sequence, and is capable of binding 2 Ni(II) with an apparent $K_D = 3.8 \mu M$. In vitro the metal affinities of Hpn and Hpn-like follow the Irving-Williams series, but in vivo the mutation of hpn and hpn-like affects sensitivity to nickel more significantly than other metals, suggesting a role in nickel sequestration. Protease digestion and chemical denaturation experiments support this in vivo selectivity of Hpn-like, as Ni(II) confers structural stability to the protein that is not observed with other metals. Double deletion of hpn and hspA leads to a more pronounced phenotypic response to nickel compared to single mutants, suggesting overlapping and redundant roles in nickel detoxification. It has been proposed that the His-rich proteins may provide a rapid detoxification response to transient nickel fluctuations before the transcriptional control of transporter expression can manifest.
1.6 Nickel-Based Genetic Regulation

1.6.1 NikR

NikR is the only known metal-regulated member of the ribbon-helix-helix family of DNA-binding proteins, characterized by intertwining N-terminal sequences of two monomers to produce an antiparallel β-strand that binds to the major groove of the DNA recognition sequence. The protein is functional as a tetramer, although there may be exceptions, with a central C-terminal MBD core connected to two flanking DNA-binding domains (DBD) through a flexible linker. NikR has been found in a wide range of archaea and eubacteria species, with *E. coli* and *H. pylori* NikR the best characterized homologs.

1.6.1.1 *E. coli* NikR

NikR was first described in *E. coli* where it acts as a nickel-dependent repressor of the nikABCDE operon. *E. coli* NikR (EcNikR, Figure 1-8) can bind one Ni(II) per monomer with a $K_D = 0.9 \text{ pM}$ (termed the high-affinity site) and additional Ni(II) with a $K_D \approx 30 \text{ nM}$ (termed the low-affinity site). The two sites allow EcNikR to bind to the palindromic operator sequence (GTATGA-N$_{16}$-TCATAC) in the nikABCDE promoter (P$_{nik}$) with two different affinities. When the high-affinity site is occupied by Ni(II), EcNikR binds DNA with a $K_D \approx 5 \text{ nM}$ and this complex tightens to a $K_D \approx 20 \text{ pM}$ upon binding of additional Ni(II). The high-affinity site of EcNikR is square planar with His87, His89, Cys95, and His76' from an adjacent monomer as ligands (Figure 1-8). Upon nickel binding, the secondary structure of EcNikR changes, with an increase in α-helix content. The crystal structure of EcNikR in the apo- and holo-forms (high-affinity site occupied) demonstrates that nickel binding induces formation of the previously unstructured α3-helix. The formation of this helix is proposed to allow several non-specific protein-DNA contacts, thereby localizing the protein to DNA where the protein can undergo a one-dimensional search for the nik promoter. Potassium ions bridge the DBD and the MBD, and are critical for Ni(II)-responsive DNA binding. The induction of tighter DNA binding occurs upon coordination of Ni(II) to the low-affinity site, proposed to consist of His48 and His110 and possibly nearby carboxylate ligands, all located between the MBD and the DBD, but some controversy exists regarding the exact location of this site. The mechanism through which this second site induces tighter DNA binding is unknown.
Despite the high-affinity site following the Irving-Williams series in terms of metal binding, nickel provides the most protection in denaturation, protease degradation, and H/D exchange experiments. This selective allosteric effect by nickel is also observed in relation to DNA-binding affinity, as Ni(II) can induce the formation of the tightest DNA-NikR complex, with the Cu(II)-NikR-DNA complex 30-fold weaker. The basis for this observed selectivity was proposed to be the square planar coordination geometry, which only Ni(II) and Cu(II) adopt. The formation of the proper coordination geometry allows for the formation of the α3-helix and subsequent DNA contacts. In vivo experiments demonstrate that NikR responds only to Ni(II), consistent with the fact that in the cell Cu(II) is reduced to Cu(I), which is incapable of forming the square planar site and triggering the required allosteric change.
1.6.1.2  *H. pylori* NikR

*H. pylori* NikR (HpNikR) shares 30% identity and 68% similarity with EcNikR, but in stark contrast to EcNikR, HpNikR acts as both an activator and repressor of a wide range of genes. Regulation by holo-HpNikR includes activation of *ureA-B* (encodes the urease structural subunits), *hpn, hpn-like, hspA*, and *nixA*, as well as repression of *frpB4, exbB/exbD, fur, nikR* and *fecA*. The diversity of regulated genes suggests that HpNikR functions as a master regulator. The consensus sequence of the HpNikR promoter has been elusive but a study examining the NikR operators in the closely related ferret pathogen, *Helicobacter mustelae*, lead to the prediction of a putative consensus sequence, TRWYA-N15-TRWYA. Despite this progress, recent studies suggest that HpNikR binding to DNA is not as simple as EcNikR, as the protein exhibits distinct conformations when bound to different promoters, a property which may be involved in DNA recognition. Furthermore, HpNikR exhibits two tiers of DNA-binding affinities for its target sequences in vitro and distinct temporal control of activated versus repressed genes in vivo. Responsiveness to pH has also been described, suggesting that HpNikR may link nickel homeostasis to acid adaptation by this organism.

Multiple crystal structures of HpNikR generated under subtly different conditions have been reported, revealing a variety of combinations of nickel sites. All of the structures have some nickel located in a square planar site similar to EcNikR, named the final (F) site, consisting of Cys107, His99, His101 and His88′ from an adjacent monomer. The second site, called the exit (X) site, consists of His74, His75, Glu104, and solvent molecules, arranged in an octahedral geometry. The third site is termed the intermediate (I) site because it contains residues from the F- and X-site. The I site is also octahedral and features His74 and His101 from one monomer, His88′ and Glu87′ from the adjacent monomer, as well as two water ligands, although slight variations have been observed. Mutation of the residues from any of these three sites disrupts the nickel response of NikR in *H. pylori*, suggesting that all three sites may be physiologically relevant, but whether differential occupancy of these sites can modulate DNA binding to the various target sequences is not clear.

The affinity with which HpNikR binds stoichiometric nickel is also a source of debate. An original $K_D = 3.5 \times 10^{-12}$ M was determined using competition experiments, yielding a number comparable to that of EcNikR. However, more recent ITC data suggest that tetrameric HpNikR
binds two nickel ions with a $K_D \approx 10$ nM and two additional ions with a $K_D \approx 100$ nM, with additional metal binding with micromolar affinities. Unlike EcNikR, there is no evidence of a DNA-binding response to excess Ni(II). Instead, HpNikR requires an additional metal ion such as magnesium, calcium, or manganese, for ureA promoter binding.223,224

1.6.2 RcnR

The RcnA Ni(II)/Co(II) exporter is controlled at the transcriptional level by the Ni(II)/Co(II)-responsive metalloregulatory protein RcnR.225 In the apo form, RcnR represses rcnA and rcnR and dissociates from the promoters upon binding nickel or cobalt, whereas Cu(II) and Zn(II) elicit a response only at much higher concentrations.227 RcnR functions as a tetramer and binds to the promoter sequence ($K_D = 255$ nM) with a large Hill coefficient of 3.3, indicating positive cooperativity for DNA binding.226

RcnR binds one equivalent of nickel per monomer with a $K_D < 25$ nM.228 Both Ni(II) and Co(II) bind to the protein in an octahedral geometry with His$_2$Cys(N/O)$_3$ ligands including the side chains of Cys35, His3, His64, and the N-terminal amine, with His3 likely serving a role in distinguishing metal ions.228,229 In addition, His60 has been proposed to act as a ligand, along with the amide backbone of the second residue.228 Although Cys35 is conserved in all RcnR homologs, this residue is dispensable for Ni(II)-responsiveness in vivo.228 Neither Ni(II) nor Co(II) appear to change the secondary structure of the protein upon binding, but both do increase protein stability.228 How this plays into the allosteric mechanism remains to be elucidated.

1.6.3 E. coli YqjI

The YqjI protein in E. coli represses the yqjH gene, which encodes a putative ferric siderophore reductase.231 YqjI releases the promoter when exposed to elevated nickel levels, which may help prevent disruption of iron homeostasis by nickel. An uncommon aspect of this regulator is the presence of a SlyD-like metal-binding domain on the N-terminus. YqjI binds two Ni(II) ions per monomer, through a mixture of His or Cys ligands, and Zn(II) was unable to displace any of the bound Ni(II).231 The extent of overlap between iron and nickel homeostasis in E. coli is not yet clear, but is reminiscent of the cross-talk between HpNikR and iron regulation in H. pylori.210
1.7 Purpose of Study

Our understanding of nickel homeostasis in *E. coli* and *H. pylori* is extensive, but there are several areas, most notably on the molecular scale, where information is lacking. One such area pertains to the GTPase activity of HypB. The function, modulation, and connection between GTPase activity and metal binding in HypB have not been well documented. In Chapter 2, the GTPase activity of *Hp*HypB is studied and a connection between enzymatic activity and metal binding is established. The metal-binding site is localized to a CH motif in *Hp*HypB and binding to this site is shown to modulate the biochemical properties of the protein. This link is further elaborated on in Chapter 3 where molecular level details are uncovered, including the role of the metal-binding ligands in signal transduction between the metal site and the GTPase active site. Furthermore, a relationship between nucleotide binding and metal binding is described, hinting at a possible role for the GTPase activity and HypB within the biosynthetic pathway of [NiFe]-hydrogenase and urease. Chapter 4 explores a GTPase from the same family as HypB, *E. coli* YjiA, and describes the relationship between metal binding in YjiA to the biochemical properties of the protein. Interestingly, the effects of metal binding to a site analogous to that in *Hp*HypB on the protein are similar to that of the HypB proteins, suggesting that these metal-regulated effects may be a family-wide trait for these GTPases. The significance of this research and a summary of the findings are noted Chapter 5, in addition to a discussion of suggested future directions research in the field should take.

An appendix to this thesis touches upon a separate research area, concerned with *EcNikR*. The appendix describes an attempt to engineer a nickel-independent variant of *EcNikR* in order ascertain the role of the α3-helix in nickel-responsive DNA binding. Unfortunately, the engineered *EcNikR* did not display nickel-independent DNA binding and it is unclear whether this is due to a flaw in the designed mutant or the absence of an effect due to the formation by the α3-helix. All together, the experiments detailed in this thesis further our knowledge of nickel homeostasis within *E. coli* and *H. pylori*, specifically on a molecular level, where previous information was lacking.
1.8 References


2 The Effects of Metal on the Biochemical Properties of Helicobacter pylori HypB, a Maturation Factor of [NiFe]-Hydrogenase and Urease

2.1 Introduction

*Helicobacter pylori* (*H. pylori*) is a gram-negative, microaerophilic bacterium that infects nearly half of the world’s population. It is responsible for chronic gastric inflammation, peptic ulcers, and atrophic gastritis, the precursor lesion to gastric cancer. Successful colonization of the human gastric mucosa by *H. pylori* requires the activity of two nickel-containing enzymes: urease and hydrogenase. Urease contains a dinuclear nickel cluster in the active site and can catalyze the hydrolysis of urea to produce ammonia and bicarbonate. The activity of urease is believed to prevent dramatic drops in the cytosolic pH of the bacterium, thus allowing it to withstand acid shock upon initial colonization of the gastric tract and thrive upon continuous exposure to milder acidic conditions. The [NiFe]-hydrogenase enzyme provides a means for *H. pylori* to utilize hydrogen gas, a byproduct of carbohydrate fermentation by other bacteria, as an energy source.

The metallocenters of urease and [NiFe]-hydrogenase are complex and require the participation of multiple dedicated accessory proteins for bioassembly. To this end, *H. pylori* contain two operons encoding the UreIEFGH and HypABCDEF accessory proteins that aid in the maturation of the urease and hydrogenase enzymes, respectively. Furthermore, gene deletion mutants in *H. pylori* revealed that interruption of either hypA or hypB not only abrogated hydrogenase activity but also disrupted urease activity. Upon nickel supplementation of the growth media both hydrogenase and urease activity can be partially restored in the mutant strains, suggesting a role for HypA and HypB in the nickel delivery steps of both biosynthetic pathways.

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Significant progress has been made in understanding the overall maturation process of the [NiFe]-hydrogenase enzyme. After the iron is incorporated along with its carbon monoxide and cyanide ligands into the large subunit of the hydrogenase precursor protein, HypA and HypB are believed to cooperate to insert the nickel ion, aided by SlyD in some organisms. The exact mechanism of this nickel delivery step is still not known, although it is clear that GTP hydrolysis by HypB is required for full hydrogenase maturation. In addition, HypB forms a complex with HypA in vitro. The latter protein contains what appears to be an intrinsic structural zinc site as well as a low-affinity nickel site (\(K_d \approx 60 \mu M\)) in a separate domain that includes the N-terminus of the protein. Several HypB proteins also bind metal at different types of sites, depending on the homolog, with the variability occurring at the N-terminus. A few HypB proteins have sequences rich in histidine residues that contribute to the binding of multiple nickel ions and are thought to function in nickel storage. Furthermore, *E. coli* HypB (EcHypB) binds stoichiometric nickel with sub-picomolar affinity to a three-cysteine motif at the N-terminus of the protein. Although this latter nickel site is essential for hydrogenase production in *E. coli*, it is not found in all HypB homologs. Finally, either nickel or zinc can bind with micromolar affinity to an essential site localized to the GTPase domain (G-domain) of EcHypB, and a crystal structure of HypB from *Methanocaldococcus jannaschii* (M. jannaschii) revealed a corresponding dinuclear zinc site bridging two monomers of a homodimer. Due to the diverse metal-binding abilities of the HypB homologs, it is possible that the mechanisms of nickel delivery to the hydrogenase enzyme may vary between organisms. However, how the metal-binding activities of the HypB proteins affect other biochemical properties and contribute to metallocenter assembly is not known.

*H. pylori* HypB (HpHypB) is of significant interest because of several unusual features. HpHypB lacks both the N-terminal high-affinity metal-binding site as well as the histidine stretch found in many other HypB homologs. Furthermore, a previous study suggested that HpHypB is incapable of binding nickel. This finding was surprising as several of the residues that comprise the metal-binding site in the G-domain of EcHypB are conserved in HpHypB (Figure 2-1). Finally, as mentioned above, HpHypB, along with HypA, also participates in urease maturation in addition to the UreDEFG accessory proteins, indicating that it has a key role in the nickel homeostasis of this pathogenic organism.
In order to learn more about the mechanism of action of this critical nickel homeostasis protein, we examined the metal-binding properties of HpHypB. Our results indicate that if the protein is fully reduced, it can bind one equivalent of nickel or zinc. The affinity of the protein for these metals was determined and the roles of two absolutely conserved Cys and His residues as metal ligands were established. Next, we investigated whether metal binding has any influence on the other attributes of HypB, including GTP hydrolysis and secondary and quaternary structures. The results demonstrate that nickel and zinc are indeed allosteric effectors, each modulating HpHypB in a distinctive fashion. This report constitutes the first detailed biochemical analysis of the effects of metal binding on the oligomeric state and enzymatic activity of a HypB protein, revealing that the activities of the protein are all intimately connected, which may be important for the function of HypB in the biosynthetic pathways of the nickel-containing urease and [NiFe]-hydrogenase enzymes.

Figure 2-1. Amino acid alignment of HpHypB with homologs. The specific strains are as follows: *Escherichia coli* K12 substr. MG1655, *Helicobacter pylori* 26695, *Helicobacter hepaticus* ATCC 51449, *Bradyrhizobium japonicum* USDA110, and *Methanocaldococcus jannaschii* DSM 2661. All sequences were retrieved from the NCBI database and aligned using ClustalW.  

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In order to learn more about the mechanism of action of this critical nickel homeostasis protein, we examined the metal-binding properties of HpHypB. Our results indicate that if the protein is fully reduced, it can bind one equivalent of nickel or zinc. The affinity of the protein for these metals was determined and the roles of two absolutely conserved Cys and His residues as metal ligands were established. Next, we investigated whether metal binding has any influence on the other attributes of HypB, including GTP hydrolysis and secondary and quaternary structures. The results demonstrate that nickel and zinc are indeed allosteric effectors, each modulating HpHypB in a distinctive fashion. This report constitutes the first detailed biochemical analysis of the effects of metal binding on the oligomeric state and enzymatic activity of a HypB protein, revealing that the activities of the protein are all intimately connected, which may be important for the function of HypB in the biosynthetic pathways of the nickel-containing urease and [NiFe]-hydrogenase enzymes.
2.2 Materials and Methods

**Materials.** Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs. Primers (Table 2-1) were purchased from Sigma Genosys. All chromatography media were from GE Healthcare. Kanamycin, tris(2-carboxyethyl)phosphine (TCEP), and isopropyl-D-1-galactopyranoside (IPTG) were purchased from BioShop (Toronto, ON). Nickel chloride salt (as a minimum, 99.9 % pure) was purchased from Sigma and the concentrations of stock solutions in water were verified by inductively coupled plasma-atomic emission spectroscopy. Other metal stocks were atomic absorption standard solutions. All other reagents were analytical or molecular biology grade from Sigma. Electronic absorption measurements were conducted on an Agilent 8453 spectrophotometer with a 1 cm pathlength. 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 vector and mutants.** The coding sequence of HpHypB was amplified from genomic *H. pylori* DNA (strain 26695) by using primers designed with restriction sites for *NdeI* (HpHypB forward) and *XhoI* (HpHypB reverse) (Table 2-1). The digested PCR product was ligated with T4 DNA ligase into the pET24b vector (Novagen) digested with *NdeI* and *XhoI* and dephosphorylated with calf intestinal phosphatase (New England Biolabs). The C106A, H107A HpHypB mutant was created from the HpHypB-pET24b construct by QuikChange PCR mutagenesis (Stratagene) with *Pfu* Turbo polymerase by using the primers listed in Table 2-1.

<table>
<thead>
<tr>
<th>Table 2-1: PCR primers used for cloning and mutagenesis.⁴</th>
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</thead>
<tbody>
<tr>
<td><strong>Primer Name</strong></td>
</tr>
<tr>
<td>HpHypB forward</td>
</tr>
<tr>
<td>HpHypB reverse</td>
</tr>
<tr>
<td>C106A, H107A forward</td>
</tr>
<tr>
<td>C106A, H107A reverse</td>
</tr>
</tbody>
</table>

⁴Restriction enzyme sites are shown in bold. Mutations are shown in lowercase.
The template strand was subsequently digested with \textit{Dpn1}. For production of large amounts of the parent \textit{HpHypB}-pET24b and mutant plasmids, the plasmids were transformed into XL-2 Blue \textit{E. coli} competent cells (Stratagene) and isolated by using the Fermentas GeneJET Plasmid Miniprep kit. All plasmids were sequenced (ACGT, Toronto, Ontario) in the forward and reverse directions by using the T7 promoter and terminator primers.

**Protein expression and purification.** For expression of wild type (WT) and mutant \textit{HpHypB}, the plasmids were transformed into BL21 Star (DE3) \textit{E. coli} cells (Invitrogen). Overnight cultures were grown and 25 mL was used to inoculate 1.5 L of LB medium supplemented with 50 μg/mL kanamycin. The cells were grown aerobically at 37 °C until the OD\textsubscript{600} reached 0.6, at which point they were induced with 0.25 mM IPTG. For some purifications, 1.5 mM NiSO\textsubscript{4} was added to the medium prior to IPTG and nickel-loaded protein was purified, but all subsequent steps were the same for both apo- or holo-proteins. After shaking at 37 °C for an additional 5 h, the cells were harvested by centrifugation and resuspended in 40 mL of 20 mM Tris(hydroxymethyl)aminomethane (Tris), pH 7.5, and 100 mM NaCl supplemented with 4 mM TCEP and two Complete, Mini, EDTA-free protease inhibitor cocktail tablets (Roche Applied Science). For a single protein purification preparation, a total of 6 L of cell culture was used. All subsequent steps were performed at 4 °C or on ice. The resuspended cells were sonicated and centrifuged at 25 000 x g for 40 min. The supernatant was passed through a 0.45 μm syringe filter and then loaded onto a DEAE Sepharose anion-exchange column (GE Healthcare) equilibrated with buffer A (20 mM Tris, pH 7.5, and 1 mM TCEP). Fractions from a NaCl gradient were screened by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12.5%). WT and mutant \textit{HpHypB} eluted at approximately 50 mM NaCl. Fractions containing the protein of interest were pooled and dialyzed against buffer A for at least 3 h. The sample was then loaded onto a HiTrapQ anion-exchange column (GE Healthcare) equilibrated with buffer A. Once again, fractions from a NaCl gradient were screened by SDS-PAGE and those containing the protein of interest (eluting at approximately 50 mM NaCl) were pooled. Following concentration of the pooled fractions to 1 mL using Amicon Ultra 3K MWCO centrifuge concentrators (Millipore), the sample was loaded onto a Superdex 200 gel filtration column (GE Healthcare) equilibrated with 25 mM HEPES, pH 7.6, 200 mM NaCl and 1 mM TCEP. Fractions containing the protein of interest were pooled and concentrated such that the final concentration was in the range of 250-500 μM. The protein concentrations were calculated
by using the extinction coefficient of 7,450 M\(^{-1}\) cm\(^{-1}\) for both WT and mutant \(Hp\)HypB at 280 nm in 4 M guanidinium HCl (GuHCl).\(^{32}\) A sample of each protein was sent for electrospray ionization mass spectrometry (ESI-MS; Department of Chemistry, University of Toronto) and the determined molecular weight of the WT and the double mutant were 27,178.0 and 27,080.0 Da, respectively. These values are 132 Da lower than the calculated molecular weights of 27,310.4 and 27,212.2 Da, suggesting that the N-terminal methionine residue is removed. All proteins were > 90% pure as estimated by Coomassie-stained SDS-PAGE that was analyzed by using the public domain NIH ImageJ program (developed at the U.S. National Institutes of Health and available on the Internet at [http://rsb.info.nih.gov/ij/](http://rsb.info.nih.gov/ij/)).

**HPLC metal analysis.** An HPLC-based method for the detection and identification of metal ions in solution was previously developed by our laboratory.\(^{33}\) For HPLC analysis, at least 50 \(\mu\)g of protein was dried by centrifugation under vacuum, reconstituted with metal-free concentrated HCl (SeaStar Chemicals), and incubated overnight at 95 °C for protein hydrolysis. The sample was once again dried to remove HCl and reconstituted in 80 \(\mu\)L of MilliQ water. This sample was injected onto a Dionex IonPak CS5A column, equilibrated with 7 mM pyridine-2,6-dicarboxylic acid, 66 mM KOH, 5.6 mM K\(_2\)SO\(_4\), and 74 mM HCOOH, attached to a metal-free Dionex BioLC HPLC system. The metals were detected at 500 nm following post-column mixing with 4-(2-pyridylazo)-resorcinol (PAR).

**Preparation of proteins.** Reduced, apo-protein was produced by incubating the protein with 10 mM EDTA and 20 mM TCEP in a Coy anaerobic glovebox at 4 °C for 48 h. The TCEP and EDTA were removed in the glovebox by exhaustive dialysis into protein buffer (25 mM HEPES, pH 7.6, 100 mM NaCl). The absence of any bound metal to the protein was confirmed by HPLC metal analysis.\(^{33}\) The free thiol content of the proteins was measured via reaction of the protein with 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB) in the presence of 6 M GuHCl and 1 mM EDTA. \(\beta\)-mercaptoethanol was used as a standard and the absorbance of the 5-mercaptopo-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 \(\mu\)M apo-\(Hp\)HypB in protein buffer and 0-120 \(\mu\)M NiCl\(_2\) were prepared in the glovebox and incubated overnight at 4 °C. The electronic absorption spectrum was monitored between 250-500 nm and corrected by
background subtraction at 600 nm. Metal stoichiometry experiments were conducted by incubating 120 µM apo-\textit{HpHypB} with either 360 µM nickel or zinc overnight at 4 °C in the glovebox. Excess metal was removed by passing the protein through a PD-10 gel filtration column (GE Healthcare) equilibrated with protein buffer in the glovebox. The protein concentration was subsequently determined (vide supra). The metal content was determined via a PAR assay in which the protein was denatured with 4 M guanidinium hydrochloride and 50 µM PAR added to the sample. The absorbance at 500 nm, corresponding to the formation a \((\text{PAR})_2\text{Me}^{2+}\) complex, was monitored and compared with a standard curve prepared with 50 µM PAR in 4 M GuHCl and known metal concentrations. To confirm the results of the PAR assay and to identify the metal present, HPLC metal analysis was also conducted on these samples.\textsuperscript{33}

\textbf{HpHypB metal affinity.} The nickel titration suggested that \textit{HpHypB} binds nickel quantitatively under the conditions described above, indicating that the metal affinity would be difficult to determine without a competitor. As such, the competitor Mag-fura-2 was selected due to its reported mid-nM \(K_d\) for nickel and zinc.\textsuperscript{35} Stocks of Mag-fura-2 (Invitrogen) were prepared in Milli-Q water and quantified by using the reported extinction coefficient of 22,000 M\(^{-1}\) cm\(^{-1}\) at 369 nm.\textsuperscript{35} To determine the metal-binding affinity of Mag-fura-2 with Ni(II) and Zn(II) under our experimental conditions, fluorescence spectroscopy was used. For determination of the Ni(II) and Zn(II) \(K_d\)s, 0 - 6 µM metal was titrated into a sample of 5 nM Mag-fura-2 in protein buffer and allowed to equilibrate at room temperature for 5 min between metal additions. Mag-fura-2 was excited at 366 nm and the decreasing fluorescence upon metal addition was monitored at 500 nm. All fluorescence experiments were conducted on a JY HORIBA Fluorolog-3 Spectrofluorometer.

Competition experiments were prepared by incubating 10 µM WT or mutant \textit{HpHypB} together with 10 µM Mag-fura-2 and varying amounts of Ni(II) or Zn(II). The samples were incubated overnight at 4 °C in the glovebox. The absorbance of Mag-fura-2 at 366 nm was monitored and the data were analyzed by using DYNAFIT\textsuperscript{36} employing a custom DYNAFIT script (Appendix II, pg. 191) that describes the competition between the protein and Mag-fura-2 for the metal.

To determine the affinity of \textit{HpHypB} for Zn(II), a competition between Zn(II) and Ni(II) was conducted. A solution containing 10 µM \textit{HpHypB} was incubated with 50 µM Ni(II) overnight at 4 °C in the glovebox. Varying concentrations of Zn(II) were then titrated into the sample and the
decrease of the 350 nm LMCT peak was monitored. The data were analyzed with DynaFit using a custom script (Appendix II, pg. 192) describing a direct competition for the protein by Ni(II) and Zn(II).

**Analytical gel filtration chromatography.** Samples containing 140 µM *Hp*HypB were incubated with the desired metal or GDP at various concentrations (see Tables 2-3 and 2-4) overnight at 4 °C in the glovebox. For samples containing GTP, the protein was incubated with GTP for at least 2 h at 4 °C in the glovebox prior to injection onto the gel filtration column. All samples contained 25 mM HEPES, pH 7.6, 100 mM NaCl and 5 mM MgCl₂. Apo-protein and metal-containing samples were loaded onto a Superdex 200 10/300 analytical gel filtration column (GE Healthcare), equilibrated with filtered and chelaxed 25 mM HEPES, pH 7.6, 200 mM NaCl, and 5 mM MgCl₂. The column was calibrated with thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B₁₂ (1.4 kDa) from BioRad. No difference in the elution profile of these standard proteins was observed when 140 µM Ni(II) or Zn(II) was added prior to gel filtration chromatography. Molecular masses were determined by plotting the log molecular masses of the standards versus the partition coefficients (Kₐᵥ), where Kₐᵥ = (Vₑ - Vₒ)/(Vₜ - Vₒ); Vₑ represents the elution volume, Vₒ is the void volume, and Vₜ is the total column volume. The eluted peaks were collected and subjected to inductively coupled plasma-mass spectrometry (ICP-MS; Department of Chemistry, University of Toronto) to ensure the presence of the added metal.

**Circular dichroism (CD) spectroscopy.** WT and mutant *Hp*HypB samples were prepared for CD spectroscopy by diluting the protein in MilliQ water to a final concentration of approximately 10 µM in the glovebox. For metal titrations, either Zn(II) or Ni(II) was added to the diluted samples and allowed to equilibrate overnight at 4 °C in the glovebox. Protein samples with GDP or GTP included 1 mM Mg(II) and were also incubated overnight at 4 °C in the glovebox. All samples were analyzed on a Jasco J-170 spectropolarimeter with a capped 1 mm pathlength cuvette in order to minimize exposure to the air. Spectra were collected at 1 nm intervals over a spectral range of 200-260 nm with a scan speed of 20 nm min⁻¹ at room temperature. The final spectra obtained are averages of six scans and corrected by subtracting the background buffer signal. The observed ellipticity was converted into mean residue ellipticity ([θ]ₘₑ; deg cm² dmol⁻¹) using the following formula:³⁷
\[ \theta_{\text{mre}} = \frac{\left( \frac{\text{MW}}{\text{N} - 1} \right) \times \theta}{[\text{Protein}] \times l \times 10} \]

where MW is the molecular weight 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 pathlength.

**GTPase assay.** GTPase activity was determined by the malachite green assay for free phosphate adapted from Lanzetta et al.\(^{38}\) A series of 160 \( \mu \)L samples containing 2 \( \mu \)M \( Hp\)HypB (in 25 mM HEPES, pH 7.6, 100 mM NaCl), 5 mM Mg(II) and varying GTP concentrations between 12.5 and 700 \( \mu \)M were incubated at 37 °C in the glovebox for 2.5 h. Controls containing only buffer, 5 mM Mg(II) and the corresponding GTP concentrations were prepared alongside the protein samples and received the same treatment. After incubation, the samples were quickly plated on a 96 well plate and 40 \( \mu \)L 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 then gently mixed by shaking the plate for 3 min, after which sodium citrate was added to a final concentration of 3.5 %. The plate was then mixed again by shaking and the color was allowed to develop for 30 min before the absorbance was measured at 630 nm with an EL808 Ultra microplate reader (Bio-Tek Instruments). 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 7.5. Samples containing nickel or zinc were incubated with the metal overnight prior to the assay (prepared as a stock of 40 \( \mu \)M protein with either 80 \( \mu \)M Zn(II) or 200 \( \mu \)M Ni(II)). These stocks were then diluted to the final protein concentration of 2 \( \mu \)M for the assay in a buffer that contained either 10 \( \mu \)M Zn(II) or 20 \( \mu \)M Ni(II).

**HpHypB structural modeling.** A homology model of \( Hp\)HypB was generated using MODELLER 9v7\(^{39}\) based on the general protocol as outlined in Eswar et al.\(^{40}\) Template searches using the \( Hp\)HypB sequence as the inquiry yielded the \( M. \) jannaschii HypB (\( Mj\)HypB) X-ray crystal structure (PDB 2HF9) as the only hit. A global alignment in MODELLER was conducted to align the two structures and this alignment was used to generate 100 models. All of these sequences featured a large unstructured loop at the N-terminus. Due to this loop, all models yielded poor pseudo-energy profiles compared to the template and PROSAII analysis\(^{41}\) indicated
that none of these models were acceptable. In order to avoid this loop region, the amino acids in
the *Hp*HypB sequence that align with the unresolved first 10 amino acids of *Mj*HypB,
corresponding to residues 1-23 of *Hp*HypB, were deleted. This sequence was then used for the
alignment and model generation as described above. The resulting models yielded better pseudo-
energy profiles and the PROSAII analysis indicated far better model quality. The best 25 models
based on their DOPE\textsuperscript{42} score were further analyzed and submitted for PROCHECK,\textsuperscript{43} ERRAT \textsuperscript{44}
and VERIFY3D\textsuperscript{45,46} analysis on the University of California at Los Angeles Structural Analysis
and Verification Server (http://nihserver.mbi.ucla.edu/SAVES/). Based on these analyses, the
best model was selected. All images were created by using UCSF Chimera.\textsuperscript{45}

### 2.3 Results

**HpHypB metal binding.** Due to the proposed role of *Hp*HypB in the bioassembly pathways of
the nickel-containing urease and hydrogenase enzymes, whether the protein could bind nickel
was the initial focus of this study. After treatment with TCEP and incubation with stoichiometric
NiCl\textsubscript{2}, an intense charge-transfer band centered at 350 nm was apparent (Figure 2-2A). This
absorption can be attributed to a Cys—S\textsuperscript{−} \rightarrow \text{Ni(II)} ligand-to-metal charge transfer (LMCT)\textsuperscript{47,48}
and suggests a contribution of at least one Cys residue as a metal ligand in *Hp*HypB. The
difference spectrum, obtained by subtracting the signal of apo-*Hp*HypB from that of *Hp*HypB
loaded with 1 equivalent of Ni(II), revealed a second peak at 293 nm (Figure 2-2A inset), which
is also attributable to a Cys—S\textsuperscript{−} \rightarrow \text{Ni(II)} LMCT.\textsuperscript{47} Upon titration of 20 µM apo-*Hp*HypB with
increasing amounts of nickel, a linear increase was observed and saturation of the signal
occurred upon the addition of 1 equivalent of nickel, suggesting a 1:1 Ni(II):*Hp*HypB
stoichiometry (Figure 2-2B). The linear portion of the titration yielded an extinction coefficient
of \((4.1 \pm 0.3) \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}\) at 350 nm. In order to confirm the metal-binding stoichiometry,
120 µM apo-*Hp*HypB was incubated with 360 µM Ni(II) followed by gel filtration
chromatography to remove excess metal. Subsequent metal analysis revealed that one equivalent
of nickel remained bound to the protein (Table 2-2). In contrast, when unreduced *Hp*HypB was
incubated with stoichiometric amounts of NiCl\textsubscript{2}, no change was evident in the electronic
absorption spectrum (Figure 2-2 inset), and the amount of nickel bound to protein was less than
20% of the protein concentration upon gel filtration and analysis with PAR. This lack of nickel
binding may be due to cysteine oxidation, which was confirmed by analysis with DTNB (data
not shown).
To measure the affinity of WT \( HpHypB \) for nickel, a competition for nickel between \( HpHypB \) and the fluorimetric metal chelator Mag-fura-2 was conducted. Mag-fura-2 was originally developed as a fluorescent dye for measuring free magnesium in the cytosol, but several studies have also used it for other divalent metals. Upon metal binding to Mag-fura-2, a blue-shift of the 366 nm absorbance to 325 nm is observed (Figure 2-3A). The affinity of Mag-fura-2 for Ni(II) has been previously reported, but under different conditions than those used in these competitions. Thus, the Mag-fura-2 nickel \( K_d \) was determined via fluorescence under our conditions and was found to be 150 ± 10 nM, a value in close agreement to that determined by Golynskiy et al. Upon titration of equivalent amounts of \( HpHypB \) and Mag-fura-2 with nickel, a decrease in the 366 nm signal of apo-Mag-fura-2 was observed, and an affinity of \( HpHypB \) for nickel (\( K_d \)) of 150 ± 20 nM was calculated (Figure 2-3B).

Figure 2-2. Nickel binding to \( HpHypB \). (A) Upon the addition of 1 equivalent of NiCl\(_2\) to apo-\( HpHypB \) (dotted line), an overall increase in absorbance is noted between 250 and 450 nm (solid line), with the formation of a new peak centered at 350 nm. A difference spectrum (inset, solid line) was generated by subtracting the signal of apo-\( HpHypB \) from that of \( HpHypB \) loaded with 1 equivalent of Ni(II). Metal analysis following gel filtration chromatography confirmed stoichiometric nickel binding (Table 2-2). When \( HpHypB \) is oxidized, the peaks in the difference spectrum upon the addition of nickel are significantly less intense (inset, dashed line), suggesting that the protein’s ability to bind Ni(II) is diminished. (B) Upon titration of 20 µM apo-\( HpHypB \) with NiCl\(_2\), a linear increase in the absorbance at 350 nm is observed until approximately 1 equivalent of metal has been added (circles). The linear region of the titration curve yields an extinction coefficient of \((4.1 \pm 0.3) \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}\). A similar titration with oxidized \( HpHypB \) does not yield an increase in the signal at 350 nm (triangles).
The affinity of \( Hp \)HypB for Ni(II). (A) In order to measure the strength of the \( Hp \)HypB-Ni(II) interaction, a competition between \( Hp \)HypB and the metal chelator Mag-fura-2 for Ni(II) was established. The spectrum of apo-Mag-fura-2 features a peak at 366 nm that blue-shifts to 325 nm upon metal binding. The spectra shown depict the change in absorbance of 10 \( \mu \)M Mag-fura-2 upon the addition of up to 50 \( \mu \)M Ni(II) in the presence of 10 \( \mu \)M \( Hp \)HypB. (B) By monitoring the decrease of the Mag-fura-2 366 nm signal upon metal binding and fitting these data versus the amount of Ni(II) added, the \( Hp \)HypB \( K_{d \text{Ni(II)}} \) was determined to be 150 \( \pm \) 20 nM. The closed circles are the average of three replicate datasets, the error bars represent the standard deviation, and the black line is the best-fit curve as determined by a custom DynaFit script.

Table 2-2: Stoichiometry of metal binding to WT and mutant \( Hp \)HypB.\(^a\)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Metal added</th>
<th>Metal bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT ( Hp )HypB</td>
<td>Ni(II)</td>
<td>1.0 ( \pm ) 0.3</td>
</tr>
<tr>
<td></td>
<td>Zn(II)</td>
<td>1.0 ( \pm ) 0.1</td>
</tr>
<tr>
<td>C106A, H107A ( Hp )HypB</td>
<td>Ni(II)</td>
<td>No metal detected</td>
</tr>
<tr>
<td></td>
<td>Zn(II)</td>
<td>1.2 ( \pm ) 0.3</td>
</tr>
</tbody>
</table>

\(^a\)Apo-\( Hp \)HypB (120 \( \mu \)M) was incubated with 360 \( \mu \)M of either Ni(II) or Zn(II) overnight at 4 °C in an anaerobic glovebox. Excess metal was removed by passing the proteins over a PD-10 gel filtration column and metal was detected either via a PAR assay or HPLC metal analysis. The data listed are average values and standard deviations of the number of metal ions bound per protein monomer from three independent experiments.
Given the clear nickel-binding ability of \textit{HpHypB} and the evidence for zinc binding to other \textit{HypB} homologs,\textsuperscript{28,30} zinc binding was also investigated. Incubation of the protein with excess zinc, followed by gel filtration chromatography and metal analysis revealed stoichiometric zinc bound (Table 2-2). When a titration of 10 µM protein was conducted with zinc in the presence of Mag-fura-2 an initial plateau region was noted in which the 366 nm signal of apo-Mag-fura-2 did not change until after 10 µM zinc was added, suggesting that the protein was outcompeting Mag-fura-2 for the available zinc (data not shown). In order to determine the zinc affinity, the ability of zinc to compete with nickel binding to \textit{HpHypB} was exploited. In this competition, \textit{HpHypB} was incubated with excess nickel and zinc was titrated into the holo-protein. As zinc bound to the protein, a decrease in the 350 nm signal of the Ni(II)-protein complex was monitored (Figure 2-4A). This experiment yielded a $K_{d, \text{Zn(II)}}$ of 1.2 ± 0.3 nM, which is two orders of magnitude tighter than the $K_{d}$ for nickel.

Finally, the CD spectrum of WT \textit{HpHypB} suggests that the protein contains significant $\alpha$-helical content as indicated by the presence of two minima at approximately 208 and 222 nm\textsuperscript{37} (data not shown). The overall secondary structure of the protein does not appear to change upon the addition of Ni(II), Zn(II) or nucleotide (data not shown).

\textbf{Identification of metal-binding residues.} Residues C106 and H107 are conserved in all \textit{HypB} homologs (Figure 2-1). In \textit{EcHypB}, these residues correspond to C166 and H167 and upon mutation to alanine, the nickel-binding ability of the protein is diminished.\textsuperscript{28} To determine if these residues participate in metal binding to \textit{HpHypB}, both C106 and H107 were mutated to alanines. The CD spectrum of the double mutant was identical to that of the WT protein, demonstrating that these mutations do not affect the secondary structure of the protein (data not shown). Mutating C106 and H107 to alanine in \textit{HpHypB} resulted in a loss of detectable nickel binding following gel filtration and metal analysis (Table 2-2), and a change in the electronic absorption spectrum was not observed upon titration with nickel (data not shown). In contrast, stoichiometric zinc binding was maintained (Table 2-2). However, zinc binding by the mutant protein was sufficiently weakened such that the affinity of the C106A, H107A \textit{HpHypB} mutant for Zn(II) could be determined via competition between the protein and Mag-fura-2. Similar to nickel, the affinity of Mag-fura-2 for zinc was first determined via fluorescence to be 101 ± 6 nM. This affinity is slightly weaker than some previously published values,\textsuperscript{35} but close to that determined by de Seny et al, who used a buffer composition similar to that of this study but at a
A clear competition was observed between Mag-fura-2 and mutant \( \text{HpHypB} \) (Figure 2-4B), yielding a \( \text{Zn(II)} \) \( K_d \) of 110 ± 40 nM. The disruption of nickel binding and weakened zinc binding upon mutation of C106 and H107, coupled to the ability of zinc to compete with nickel, suggests that the coordination spheres of the two metals share some ligands but are not identical.

\textbf{HpHypB quaternary structure.} Analytical gel filtration chromatography was used to investigate the oligomeric state of apo-\( \text{HpHypB} \) as well as how it is affected by metal and/or nucleotide. In the apo form, \( \text{HpHypB} \) eluted as a monomer from the column (Figure 2-5). A previous study reported that \( \text{HpHypB} \) exists as a homodimer in solution\(^ {21} \) and this discrepancy may be due to different experimental conditions such as protein oxidation. Upon addition of half an equivalent of nickel, a significant portion of the protein dimerizes, with all of the protein existing as a dimer upon the addition of one equivalent of nickel (Figure 2-5). Further addition of lower pH.\(^ {50} \)
nickel did not change the dimeric state (data not shown). When the conserved residues C106 and H107 were mutated to alanine, the protein lost the ability to dimerize upon the addition of nickel (Table 2-3), indicating that these residues are necessary for metal-induced dimerization of the protein. Zinc, on the other hand, did not affect the protein’s oligomeric state (Figure 2-5). Even with 420 µM Zn(II) in the mobile phase, the protein still eluted as a monomer (data not shown). The presence of metal in all holo-samples following elution from the column was confirmed by ICP-MS. When the protein was incubated with equimolar amounts of zinc and nickel, little dimerization was observed (Table 2-3). ICP-MS analysis indicated that zinc was bound to the protein instead of nickel, supporting the finding that HpHypB has a higher affinity for zinc than nickel and that it preferentially binds zinc over nickel.

Figure 2-5. The effect of metal on the quaternary structure of HpHypB. In the absence of metal, HpHypB (140 µM) elutes from a gel filtration column at a volume corresponding to a monomer (—). Upon the addition of half an equivalent of nickel (---), a significant proportion of the protein dimerizes, with all protein eluting as dimer upon the addition of a single equivalent of metal (---). The addition of 1 equivalent of zinc did not affect the protein’s oligomeric state (•••). The chromatographic traces shown are representative experiments using a Superdex 200 10/300 analytical column with 25 mM HEPES, pH 7.6, 200 mM NaCl, and 5 mM MgCl₂ as the mobile phase. The eluate was monitored at 280 nm and the nickel-bound protein absorbs much more strongly at this wavelength than apo-protein (Figure 2-2). The ticks at the top of the graph denote the elution volumes of the protein standards. From left to right, the identity of the standards and their respective molecular masses are as follows: γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and Vitamin B₁₂ (1.4 kDa).
Table 2-3: Summary of gel filtration chromatography results of WT and mutant *HpHypB* with added metals.$^a$

<table>
<thead>
<tr>
<th>Protein</th>
<th>Added Metal</th>
<th>Elution Volume (mL)</th>
<th>Calculated MW (kDa)</th>
<th>Relative Peak Area$^b$</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT <em>HpHypB</em></td>
<td>Apo</td>
<td>16.9 ± 0.2</td>
<td>28.1 ± 2.6</td>
<td>100</td>
<td>Monomer</td>
</tr>
<tr>
<td></td>
<td>0.5 eq. Ni(II)</td>
<td>15.9 ± 0.1</td>
<td>44.7 ± 1.6</td>
<td>76</td>
<td>Dimer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17.2 ± 0.1</td>
<td>23.0 ± 0.7</td>
<td>24</td>
<td>Monomer</td>
</tr>
<tr>
<td></td>
<td>1 eq. Ni(II)</td>
<td>15.7 ± 0.1</td>
<td>50.9 ± 1.9</td>
<td>100</td>
<td>Dimer</td>
</tr>
<tr>
<td></td>
<td>1 eq. Zn(II)</td>
<td>15.6 ± 0.1</td>
<td>52.7 ± 2.7</td>
<td>3.5</td>
<td>Dimer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17.0 ± 0.1</td>
<td>25.8 ± 1.0</td>
<td>96.5</td>
<td>Monomer</td>
</tr>
<tr>
<td></td>
<td>1 eq. Zn(II) + 1 eq. Ni(II)</td>
<td>15.86 ± 0.01</td>
<td>46.2 ± 0.2</td>
<td>5</td>
<td>Dimer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17.34 ± 0.02</td>
<td>21.9 ± 0.2</td>
<td>95</td>
<td>Monomer</td>
</tr>
<tr>
<td>C106A, H107A <em>HpHypB</em></td>
<td>Apo</td>
<td>17.2 ± 0.1</td>
<td>23.8 ± 0.8</td>
<td>100</td>
<td>Monomer</td>
</tr>
<tr>
<td></td>
<td>1 eq. Ni(II)</td>
<td>17.1 ± 0.1</td>
<td>24.5 ± 0.8</td>
<td>100</td>
<td>Monomer</td>
</tr>
<tr>
<td></td>
<td>1 eq. Zn(II)</td>
<td>17.11 ± 0.01</td>
<td>24.6 ± 0.1</td>
<td>100</td>
<td>Monomer</td>
</tr>
</tbody>
</table>

$^a$Errors are standard deviations of at least two replicates. The expected molecular weights are: WT *HpHypB* monomer: 27.2 kDa; WT *HpHypB* dimer: 54.4 kDa; C106A, H107A *HpHypB* monomer: 27.1 kDa.

$^b$The relative peak areas varied by < 3.5 % of the total peak area.
Table 2-4: Summary of gel filtration chromatography results of WT *HpHypB* with added nucleotides.\(^a\)

<table>
<thead>
<tr>
<th>Added ligand</th>
<th>Elution Volume (mL)</th>
<th>Calculated MW (kDa)</th>
<th>Relative Peak Area(^b)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 eq. GTP</td>
<td>15.9 ± 0.2</td>
<td>45.4 ± 4.0</td>
<td>8</td>
<td>Dimer</td>
</tr>
<tr>
<td></td>
<td>17.2 ± 0.3</td>
<td>24.0 ± 3.2</td>
<td>92</td>
<td>Monomer</td>
</tr>
<tr>
<td>5 eq. GDP</td>
<td>16.0 ± 0.1</td>
<td>43.9 ± 1.7</td>
<td>9.5</td>
<td>Dimer</td>
</tr>
<tr>
<td></td>
<td>17.4 ± 0.1</td>
<td>21.3 ± 0.7</td>
<td>90.5</td>
<td>Monomer</td>
</tr>
<tr>
<td>1 eq. Ni(II) + 5 eq. GDP</td>
<td>15.8 ± 0.2</td>
<td>48.0 ± 4.4</td>
<td>81.5</td>
<td>Dimer</td>
</tr>
<tr>
<td></td>
<td>17.21 ± 0.04</td>
<td>23.4 ± 0.4</td>
<td>18.5</td>
<td>Monomer</td>
</tr>
<tr>
<td>1 eq. Ni(II) + 5 eq. GTP</td>
<td>16.03 ± 0.04</td>
<td>42.4 ± 8.0</td>
<td>84</td>
<td>Dimer</td>
</tr>
<tr>
<td></td>
<td>17.29 ± 0.03</td>
<td>22.4 ± 0.3</td>
<td>16</td>
<td>Monomer</td>
</tr>
<tr>
<td>5 eq. GTP + 400 μM GTP in MP</td>
<td>16.8 ± 0.1</td>
<td>58.4 ± 3.5</td>
<td>31.5</td>
<td>Dimer</td>
</tr>
<tr>
<td></td>
<td>18.34 ± 0.04</td>
<td>27.6 ± 0.6</td>
<td>68.5</td>
<td>Monomer</td>
</tr>
<tr>
<td>5 eq. GDP + 400 μM GDP in MP</td>
<td>16.99 ± 0.01</td>
<td>54.2 ± 0.4</td>
<td>27</td>
<td>Dimer</td>
</tr>
<tr>
<td></td>
<td>18.3 ± 0.1</td>
<td>28.1 ± 2.0</td>
<td>73</td>
<td>Monomer</td>
</tr>
<tr>
<td>5 eq. GTP + 400 μM GTP in MP + 1 eq. Zn(II)</td>
<td>18.33 ± 0.04</td>
<td>27.9 ± 0.5</td>
<td>100</td>
<td>Monomer</td>
</tr>
<tr>
<td>5 eq. GDP + 400 μM GDP in MP + 1 eq. Zn(II)</td>
<td>18.225 ± 0.007</td>
<td>29.3 ± 0.1</td>
<td>100</td>
<td>Monomer</td>
</tr>
</tbody>
</table>

\(^a\)Errors are standard deviations of at least two replicates. The expected molecular weights are: WT *HpHypB* monomer; 27.2 kDa; WT *HpHypB* dimer; 54.4 kDa. MP indicates that the nucleotide was included in the mobile phase.

\(^b\)The relative peak areas varied by < 3.5 % of the total peak area.
The calculated molecular weight of the apo-HpHypB species from the gel filtration chromatography experiments (28.1 ± 2.6 kDa) is in close agreement with the predicted molecular weight (27.2 kDa). However, the monomeric species in the presence of metal elutes as a slightly smaller protein (23.0 - 25.8 kDa), suggesting the formation of a more compact protein structure upon binding of metal. Furthermore, the observed molecular weight of the nickel-induced dimeric species is smaller than expected (MW\textsubscript{obs}= 50.9 ± 1.9 kDa, MW\textsubscript{pred}= 54.2 kDa), suggesting that the metal-bound dimer is also more compact in its overall shape.

If the protein was incubated with nucleotides and applied to a gel filtration column, little change in the oligomeric state of the protein was observed (Table 2-4). However, when 400 µM GDP or GTP was included in the mobile phase, some nucleotide-dependent dimerization of HpHypB was detected (Table 2-4). The requirement of additional nucleotides in the mobile phase suggests that the interaction between the protein and nucleotide is weak enough for dissociation to occur during the time scale of the gel filtration experiment. GTP hydrolysis by HpHypB is slow enough (\textit{vide infra}) such that over the course of the gel filtration experiment significant hydrolysis of the GTP to GDP should not be an issue. Finally, the addition of zinc to the protein inhibited both GTP- and GDP-dependent dimerization (Table 2-4).

**GTPase activity.** As previously reported,\textsuperscript{21} HpHypB catalyzes the hydrolysis of GTP (Figure 2-6 and Table 2-5). The experiments shown here reveal that the enzymatic activity of HpHypB is quite low, with a $k_{cat}$ of only $(6 ± 1) \times 10^{-4}$ s\(^{-1}\) and a $K_M$ of $(5 ± 1) \times 10^{-5}$ M ($k_{cat}/K_M$ of $12 ± 3$ M\(^{-1}\) s\(^{-1}\$). To determine if metal binding modulates the GTPase kinetics, the protein was incubated with extra nickel or zinc. In the presence of Ni(II), the $k_{cat}$ increases several fold to $(1.6 ± 0.1) \times 10^{-3}$ s\(^{-1}\) and the $K_M$ of the protein increases to $(1.6 ± 0.5) \times 10^{-4}$ M, suggesting a slightly weakened affinity for GTP compared to that of apo-HpHypB. Overall, the addition of Ni(II) to the protein does not affect the $k_{cat}/K_M$, as it remains at $11 ± 3$ M\(^{-1}\) s\(^{-1}\$. In contrast, the addition of Zn(II) inhibits the enzymatic activity and no activity was detectable in the presence of 10 µM Zn(II) (Figure 2-6). Mutating the C106 and H107 residues to alanine did not dramatically affect GTPase activity, and the double mutant had a $K_M = (4.0 ± 0.2) \times 10^{-5}$ M, $k_{cat} = (1.0 ± 0.1) \times 10^{-4}$ s\(^{-1}\), and $k_{cat}/K_M = 2.4 ± 0.3$ M\(^{-1}\) s\(^{-1}\$ (data not shown).
Figure 2-6. The GTPase activity of *HpHypB*. Apo-*HpHypB* (2 μM, squares) was incubated with increasing concentrations of GTP at 37 °C for 2 h in an anaerobic glovebox prior to detection of released inorganic phosphate by using a colorimetric assay. Fitting the data from several experiments to the Michaelis-Menten equation yields a $k_{cat}$ of $(6 \pm 1) \times 10^{-4}$ s$^{-1}$, a $K_M$ of $(5 \pm 1) \times 10^{-5}$ M, and a $k_{cat}/K_M$ of $12 \pm 3$ M$^{-1}$ s$^{-1}$. The presence of 20 μM nickel (circles) results in an increased $k_{cat}$ of $(1.6 \pm 0.1) \times 10^{-3}$ s$^{-1}$ and $K_M$ of $(1.6 \pm 0.5) \times 10^{-4}$ M. Thus, overall the $k_{cat}/K_M$ does not change and remains at $11 \pm 3$ M$^{-1}$ s$^{-1}$. The addition of 10 μM zinc inhibits GTPase activity (triangles). All datasets are the average of at least three independent replicates and error bars represent the standard deviation of the replicates.

### Table 2-5: Kinetics of GTP hydrolysis by *HpHypB*.a

<table>
<thead>
<tr>
<th></th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_M$ (M)</th>
<th>$k_{cat}/K_M$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo WT <em>HpHypB</em></td>
<td>$(6 \pm 1) \times 10^{-4}$</td>
<td>$(5 \pm 1) \times 10^{-5}$</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>+ 20 μM Ni(II)</td>
<td>$(1.6 \pm 0.1) \times 10^{-3}$</td>
<td>$(1.6 \pm 0.5) \times 10^{-4}$</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>+ 10 μM Zn(II)</td>
<td>No measurable hydrolysis</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*aAll GTPase assays were conducted with 2 μM *HpHypB* in protein buffer supplemented with 5 mM MgCl$_2$. Samples containing metal were pre-incubated with either zinc or nickel overnight at 4°C in an anaerobic glovebox. The amount of released phosphate was detected using a modified Malachite Green assay. The data listed are average values and standard deviations from three independent experiments.*
**HpHypB structural modeling.** The crystal structure of *MjHypB* provided an excellent template upon which a structural model of *HpHypB* could be constructed. The sequences of *HpHypB* and *MjHypB* are 30% identical and 51% similar, sufficient to create a reasonable structural model of *HpHypB*. In order to verify the validity of our final homology model, several tests were conducted. ERRAT analyzes the non-bonded interactions between different atoms and scores the overall structure based on a comparison to highly refined structures. The final model had an ERRAT score of 71.09 indicating an acceptable model (score > 50). Furthermore, PROCHECK, an analysis of the overall stereochemistry, indicated no major deviations from ideal protein stereochemistry, with 100% of the residues falling within acceptable regions of the Ramachandran plot. VERIFY 3D analyzes the compatibility of a model with its own amino acid sequence. For the *HpHypB* homology model, 82.27% of the residues have a score of greater than 0.2, indicating a good quality model. The calculated Cα RMSD between the homology model and the template was 0.285 Å, demonstrating close homology between the model and template.

The constructed model of *HpHypB* is a globular protein with a 7-strand parallel β-sheet core surrounded by α-helices, a structure characteristic of P-loop NTPases of the SIMIBI class (Figure 2-7). This classification is in accordance with the large P-loop NTPase phylogenetic study conducted by Leipe et al. in which the authors found that the HypB proteins are evolutionarily related to the UreG, COG0523 (also known as CobW), and ArgK proteins, which together comprise the G3E family of GTPases. This family is defined by a glutamate residue in the Walker B motif (hhhhEXXG where h is any hydrophobic amino acid; also referred to as the G3 motif) which substitutes for the conserved Mg(II)-binding aspartate residue (hence the name G3E). An alignment of the *HpHypB* and *MjHypB* sequences (Figure 2-1) reveals a conservation of all the major GTPase motifs, as well as the CH motif located between the Walker A and B motifs. In the homology model, these two residues are located on the surface of the protein (Figure 2-7) adjacent to an additional conserved cysteine, C142. The surface-exposed position of these residues is ideal for mediating nickel-dependent dimerization. Furthermore, an overlay of the protein model with that of the crystal structure of *MjHypB* demonstrates that the major nucleotide motifs are located in similar positions as would be expected (data not shown).
2.4 Discussion

*HpHypB* contributes to the maturation of both urease and hydrogenase in *H. pylori*. Initial characterization of the protein suggested that it does not bind Ni(II), however the experiments reported here demonstrate that *HpHypB* can bind both nickel and zinc when fully reduced. The protein is clearly sensitive to oxygen, but given that hydrogenase enzymes involved in utilizing H₂ as an energy source are typically associated with anaerobic metabolism, this oxygen sensitivity is likely not an issue for *HpHypB in vivo*. It is possible that the disruption of metal binding by oxidation is a mechanism to regulate the function of *HpHypB* and hydrogenase and/or urease production in *H. pylori*, as has been suggested for other types of systems (for examples see 55-57), but there is no evidence yet to support this hypothesis.

Although *HpHypB* lacks both the N-terminal high-affinity metal-binding sequence and a histidine-rich sequence, it is still capable of binding stoichiometric metal. The fact that zinc can compete with nickel suggests that the metal ions have some common ligands. It is also possible that binding of zinc at a separate location of *HpHypB* has an allosteric effect on the conformation...
of the protein that inhibits nickel binding, but the lack of any detectable change in the CD spectrum upon binding of either metal does not support a dramatic change in the protein conformation. Furthermore, mutating the conserved C106/H107 amino acids completely abrogates detectable nickel binding and weakens the affinity for zinc by two orders of magnitude, indicating that both residues can coordinate either nickel or zinc. However, the distinct effects of each type of metal ion on the properties of the protein indicate that the sites are not superimposable. Upon closer examination of the homology model of HpHypB, very few residues located near the CH motif appear to be capable of binding metals. One possibility is C142, located adjacent to the CH motif (Figure 2-7). Preliminary experiments with a C142A mutant suggested that nickel binding is significantly weakened when this residue is removed, but extensive experimentation was hindered due to metal-induced protein aggregation of the mutant protein (data not shown). This cysteine residue is conserved in all HypB homologs, suggesting a critical role in the protein (Figure 2-1). A second possible metal-ligand is E109, located near H107 (Figure 2-7). This residue is also well-conserved in HypB homologs, either as a glutamic acid or an aspartic acid (Figure 2-1).

The conserved CH motif serves to bind metal in the MjHypB crystal structure, which revealed a bi-metallic site at the interface between two monomers. The presence of a similar non-symmetrical site in the isolated G-domain of EcHypB loaded with zinc was suggested by X-ray absorption spectroscopy. However, despite the evidence for a metal-binding site at a dimeric interface, no prior biochemical study has investigated the effect of metal on the oligomeric structure of HypB. In the case of HpHypB, metal-induced dimerization of HpHypB is not observed in the presence of zinc, and in fact zinc blocks dimerization stimulated by nucleotide, indicating that zinc binding at a dimer interface is not conserved. Dimerization is only induced by nickel, further supporting the differences between the metal coordination properties of each metal.

The disparate effects of nickel and zinc on the quaternary structure of HpHypB may explain the metal-dependent modulation of the GTPase activity observed in this study. The structure of MjHypB revealed nucleotide-binding sites composed of residues from both monomers, suggesting that the dimer is the form required for GTP hydrolysis. Thus a metal that enhanced dimerization, such as nickel in the case of HpHypB, would either activate the GTPase or have no effect, whereas metal binding that blocked dimerization would inhibit hydrolysis, as observed
upon addition of zinc. However, the situation might not be so straightforward because the quaternary structure required for active \(Hp\)HypB has not yet been established, and work with full-length \(Ec\)HypB indicates that the protein is active as a monomer.\(^{58}\) In support of an alternative explanation, the invariant C142, located at an appropriate position to coordinate a metal ion bound to C106/H107 on the monomeric protein (Figure 2-7), is within the highly-conserved Switch II motif (Figure 2-1) that connects GTP hydrolysis to conformational changes in the protein.\(^{25}\) Given this location of C142, it is feasible that metal binding to \(Hp\)HypB could influence GTP hydrolysis in a manner that depends on if and/or how C142 coordinates the metal but that is independent of quaternary structure.

It has been shown that several isolated HypB proteins are poor GTPases, and \(Hp\)HypB is no exception.\(^{25,26,59}\) This low level of hydrolysis is typical of GTPases that are examined \textit{in vitro} where they lack additional protein partners and cofactors that may stimulate GTPase activity \textit{in vivo}.\(^{60}\) For example, mammalian p21\textsuperscript{ras}, one of the best characterized GTPases involved in control of critical regulatory pathways such as cell proliferation and differentiation,\(^{61}\) has low GTPase activity \textit{in vitro}, with a \(k_{cat}\) of \(3.4 \times 10^{-4}\) s\(^{-1}\),\(^{62}\) a number comparable to that of \(Hp\)HypB. Given the metal-binding activity of the protein, it was reasonable to predict that metal could activate GTP hydrolysis. However, nickel binding has a minimal effect and zinc regulates catalysis in a negative manner. Thus it is likely that the GTPase activity of \(Hp\)HypB is activated in the context of a functional maturation pathway through the interaction with another protein or cofactor. So far, \(Hp\)HypB has been shown to also interact with HypA \textit{in vitro},\(^{21}\) as well as with SlyD and UreG \textit{in vivo},\(^{19}\) but it may also be a part of larger, multi-component complexes that cooperate for hydrogenase and urease assembly. Studies conducted by Mehta et al. demonstrated that in vitro, addition of equimolar amounts of HypA fail to alter the GTPase activity of \(Hp\)HypB.\(^{21}\) Further work is needed to determine if any of these additional proteins activates GTPase turnover of \(Hp\)HypB.

The GTPase activity of \(Hp\)HypB is critical for hydrogenase and urease production in \textit{H. pylori}.\(^{19}\) Upon mutation of K59, a critical residue involved in nucleotide binding, to alanine, the GTPase activity of the protein is nearly abolished\(^{21}\) and hydrogenase and urease production is hindered.\(^{19}\) Supplementation of the media with nickel partially restored hydrogenase activity and fully restored urease activity, suggesting a role of \(Hp\)HypB in the nickel-insertion steps of these two maturation pathways.\(^{19}\) The participation of metal-binding NTPases in the biosynthetic pathways
of complex metallocenters is an emerging trend,\textsuperscript{16} although whether a common theme of metal-regulated NTP hydrolysis exists is not yet clear. For example, metal-dependent dimerization was observed for CooC,\textsuperscript{63,64} the ATPase involved in carbon monoxide dehydrogenase biosynthesis, but ATP hydrolysis was not dramatically affected by the addition of nickel. Furthermore, the GTPase dedicated to urease maturation, UreG, binds nickel or zinc with micromolar affinities at a site that likely includes the CxH motif found at a similar location with respect to the GTPase motifs as C106/H107 of \textit{HpHypB}.\textsuperscript{65-67} However, the relative affinities reported for nickel versus zinc, and whether the metals affect quaternary structure, vary depending on the UreG homolog. Finally, comparative genomic analysis of the COG0523 proteins, another part of the G3E family of P-loop GTPases that also includes HypB and UreG, lead to the prediction that several subgroups have roles in metal metabolism with some of them linked to zinc homeostasis,\textsuperscript{68} so it is likely that metal binding is a common property of the G3E GTPases that contributes to pathways including, but not limited to, metallocenter assembly.

\textit{HpHypB} lacks the high-affinity metal-binding sequence found at the N-terminus of \textit{EcHypB}, a site postulated to be dispensable in various organisms and only required under certain growth conditions.\textsuperscript{28} On the other hand, the affinities of the metal site in \textit{HpHypB} are at least several orders of magnitude tighter than the corresponding site in the G-domain of \textit{EcHypB}, perhaps indicating adaptation of the respective metal-binding sites to the specific conditions of each organism. Along the same lines, \textit{H. pylori} expresses several other proteins that bind multiple nickel ions and appear to be involved in the maturation pathway of hydrogenase and/or urease, including the GroES homolog HspA, two small polypeptides called Hpn and Hpn-like, and the peptidyl-prolyl isomerase SlyD.\textsuperscript{13,16} These additional proteins have regions rich in various combinations of metal-binding amino acids and can bind multiple nickel ions, which may compensate for the lack of a His-rich region in either \textit{HpHypB} or the \textit{H. pylori} UreE protein.\textsuperscript{16} Furthermore, the reduced nickel-binding capacity of \textit{H. pylori} UreE has been suggested to be a plausible reason for the supplemental involvement of \textit{HpHypB} in urease bioassembly.\textsuperscript{69} In support of this hypothesis, \textit{H. pylori} UreE fused to an extra His-rich sequence can rescue the urease activity in \textit{H. pylori hypA} and \textit{hypB} deletion mutants.\textsuperscript{70}

The results presented here demonstrate that \textit{HpHypB} binds stoichiometric nickel or zinc, and that metal modulates the GTPase activity and dimerization state, revealing for the first time a connection between metal binding and other activities of a HypB protein. However, which metal
is physiologically relevant or whether both metals have a role is not yet clear. In the context of EcHypB, Leach et al. speculated that a metal site in the G-domain could be responsible for detecting nickel delivery to the hydrogenase precursor protein and accelerating GTP hydrolysis, thus activating the next stage in the process such as disengagement with the other factors of the nickel-insertion complex. However, nickel binding does not dramatically enhance the GTP hydrolysis activity of HpHypB, so other consequences of the metal-protein interactions must be considered. Alternatively, perhaps control of the formation of the HpHypB dimer is a regulatory mechanism that modulates interactions with other proteins. In the holo-homodimer state, nickel binding to HpHypB could act as an “off” switch, thereby preventing any further interactions with partner proteins such as HypA. However, further research to elucidate the effect of metal on the formation of the HypA-HpHypB heterodimer relative to that of the HpHypB homodimer is required. Another non-exclusive method of control could also be achieved in the zinc-bound state because the activity of the protein is significantly inhibited upon the addition of zinc. In the zinc-bound state, the enzyme’s GTPase activity is turned off but can be restored by metal removal or replacement of the zinc by nickel. Perhaps other factors involved in hydrogenase bioassembly, such as SlyD, are required for this zinc removal and only when HpHypB is required for urease or hydrogenase maturation is the GTPase activity turned on in this manner. Whether this metal-dependent control of GTPase activity is relevant in vivo is unclear, but it does highlight an interesting mechanism through which the enzyme’s activity may be regulated. The distinct effects of nickel versus zinc may reflect regulation of the protein by metal availability in the H. pylori cytoplasm, which could change depending on the needs of the cell and signal an increase or decrease in nickel enzyme requirements. Further elucidation of this metal-dependent GTPase activity is required to understand what role it plays in the nickel insertion process of hydrogenase and urease bioassembly.
2.5 References


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proteins HypA and HypB for full activity of both hydrogenase and urease in *Helicobacter 


3 Relationship Between Selective Metal Coordination and Nucleotide Binding by *Helicobacter pylori* HypB

3.1 Introduction

*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. Critical to *H. pylori*’s ability to colonize the human gastric mucosa are two Ni(II)-containing enzymes: urease and [NiFe]-hydrogenase. Urease is responsible for preventing dramatic drops in the cytosolic pH by catalyzing the hydrolysis of urea to produce buffering ammonia and bicarbonate. [NiFe]-hydrogenase provides *H. pylori* with an energy source by catalyzing the oxidation of molecular hydrogen, a byproduct of carbohydrate fermentation by other gut microorganisms. Both enzymes require multiple dedicated accessory proteins for biosynthesis of their intricate metallocenters. The *H. pylori* genome contains two operons encoding the UreEFGH and HypABCDEF accessory proteins responsible for the maturation of urease and [NiFe]-hydrogenase, respectively. Furthermore, the study of gene-directed mutants in *H. pylori* demonstrated that HypA and HypB bridge the two pathways because they are involved in the maturation of urease as well as [NiFe]-hydrogenase. The enzymatic deficiencies of the mutant strains are at least partially restored by the addition of high concentrations of nickel to the growth media, suggesting that these proteins are required for Ni(II) delivery during the production of the metalloenzymes urease and [NiFe]-hydrogenase.

The maturation 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. Next, HypA and HypB are believed to cooperate to insert the nickel ion, in a process that requires the GTPase activity of HypB and that is aided by SlyD in some organisms. HypA can form a complex with HypB and SlyD in the absence of the hydrogenase large subunit, and there is evidence that HypA docks this

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This chapter is a draft of a manuscript to be submitted to the Journal of Biological Chemistry. The authors on the manuscript are A.M. Sydor, H. Lebrette, R. Ariyakumaran, C. Cavazza, and D.B. Zamble. Author contributions: A.M.S. performed the experiments except for the crystallography, analyzed the data, and wrote the paper. R.A. assisted A.M.S. with the experiments. H.L. and C.C. conducted the crystallography, analyzed the resulting data, and wrote the paper. D.B.Z. wrote the paper.
pre-assembled nickel insertion complex onto the [NiFe]-hydrogenase precursor protein.\textsuperscript{27} Furthermore, it has been proposed that HypA is responsible for steering Ni(II) to either the urease or hydrogenase maturation pathway.\textsuperscript{28}

There are numerous HypB homologs with varying metal-binding abilities,\textsuperscript{9,26,29,30} 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.\textsuperscript{22,31} Mutation of several of the conserved residues that serve as metal ligands in \textit{E. coli} HypB (\textit{EcHypB}) abrogates hydrogenase production in this organism, indicating the critical role of this metal-binding site of HypB during metallocenter assembly.\textsuperscript{32} This is the only metal site identified in \textit{H. pylori} HypB (\textit{HpHypB}), which can bind one nickel ion with a $K_D$ of 150 nM.\textsuperscript{9} However, zinc binds with an affinity that is about two orders of magnitude tighter, prompting speculation about the identity of the physiologically relevant metal.\textsuperscript{9} The only crystal structure of metal-loaded HypB is from \textit{Methanocaldococcus jannaschii}, which revealed a dinuclear asymmetric zinc site at the interface of a protein dimer, with the first metal ion bound by water and three Cys residues, one of which bridges to a second Zn(II) bound by another Cys, one His, and one water.\textsuperscript{31} The observation that Zn(II) binding to \textit{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,\textsuperscript{9} but little was known how these key cofactors of HypB impact each other.

In this study, we investigated the relationship between metal binding and the GTPase cycle of \textit{HpHypB}. Nucleotide alters the nickel, but not zinc, stoichiometry and also modulates the affinity of the \textit{HpHypB}-nickel complex. Structural analysis of nucleotide-loaded \textit{HpHypB} reveals 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 \textit{HpHypB} monomers, a coordination environment distinct from that predicted in the absence of nucleotide.\textsuperscript{9,29,31,32} In support of this switch in metal coordination, biochemical examination of His107 suggests 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 Cys142, a metal ligand that is donated from the signal transducing Switch II GTPase motif. Altogether, our results demonstrate that the nucleotide bound state of the protein and metal coordination directly influence each other, and this has important implications on the proposed role of \textit{HpHypB} in the maturation of urease and [NiFe]-hydrogenase.
3.2 Materials and Methods

**Materials.** Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs. Primers (Table 3-1) were purchased from Sigma Genosys. All chromatography media were from GE Healthcare. Kanamycin, tris(2-carboxyethyl)phosphine (TCEP), isopropyl β-D-1-thiogalactopyranoside (IPTG), and ethylenediaminetetraacetic acid (EDTA) were purchased from BioShop (Toronto, ON). Nickel chloride salt (as a minimum, 99.9 % pure) was purchased from Sigma-Aldrich and the concentrations of stock solutions in water were verified by inductively coupled plasma-atomic emission spectroscopy. The GTP analogs used in this study were guanosine 5′-[^β,γ-imido]triphosphate (GDPNP) and guanosine 5′-O-[γ-thio]triphosphate (GTPγS) from Sigma-Aldrich. 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 pathlength. All fluorescence experiments were conducted on a JY HORIBA Fluorolog-3 Spectrofluorometer. 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 previously described. The H107A and C142S mutations were introduced into the *HpHypB*-pET24b construct by QuikChange PCR mutagenesis (Stratagene) with Pfu polymerase using the primers listed in Table 3-1. The template strand was subsequently digested with *Dpn*1. 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

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>HpHypB</em> C142S forward</td>
<td>5'CGTGGGGGAATTTGGTTTcCCCCTCAAGCTATAATCTAG3'</td>
</tr>
<tr>
<td><em>HpHypB</em> C142S reverse</td>
<td>5'CTAGATTATAGCTTGAGGGGgAAACCAAATTCCCCACG3'</td>
</tr>
<tr>
<td><em>HpHypB</em> H107A forward</td>
<td>5'CCACCAGGCGAAGCATGcTTTGAAGCGAGC3'</td>
</tr>
<tr>
<td><em>HpHypB</em> H107A reverse</td>
<td>5'GCTCGCTTCCAAAgeGCATGCTTGCAGGCAGTG3'</td>
</tr>
</tbody>
</table>

*Mutations are shown in lowercase.
GeneJET Plasmid Miniprep kit. All plasmids were sequenced (ACGT, Toronto, Ontario) in the forward and reverse directions by using the T7 promoter and terminator primers.

For protein purification, overnight cultures were grown and 25 mL was used to inoculate 1.5 L of LB medium supplemented with 50 µg/mL kanamycin. The cells were grown aerobically at 37 °C until the A₆₀₀ reached 0.6, at which point 1.5 mM NiSO₄ was added to the medium and 30 min later protein expression was induced with 0.25 mM IPTG. After shaking at 37 °C for an additional 5 h, the cells were harvested by centrifugation and resuspended in 40 mL of 20 mM Tris(hydroxymethyl)aminomethane (Tris), pH 7.5, and 100 mM NaCl supplemented with 4 mM TCEP and three Complete, Mini, EDTA-free protease inhibitor cocktail tablets (Roche Applied Science). All subsequent steps were performed at 4 °C or on ice. The resuspended cells were sonicated and centrifuged at 25 000 x g for 40 min. The supernatant was passed through a 0.45 µm syringe filter and then loaded onto a DEAE Sepharose anion-exchange column (GE Healthcare) equilibrated with buffer A (20 mM Tris, pH 7.5, and 1 mM TCEP). Fractions from a NaCl gradient were screened by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12.5 %). WT and mutant HpHypB eluted at approximately 50 mM NaCl. Fractions containing the protein of interest were pooled and dialyzed against buffer A for at least 3 h. The sample was then loaded onto a HiTrapQ anion-exchange column (GE Healthcare) equilibrated with buffer A. Once again, fractions from a NaCl gradient were screened by SDS-PAGE and those containing the protein of interest (eluting at approximately 50 mM NaCl) were pooled. Following concentration of the pooled fractions to 1 mL using Amicon Ultra 3K MWCO centrifuge concentrators (Millipore), the sample was loaded onto a Superdex 200 gel filtration column (GE Healthcare) equilibrated with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.6, 200 mM NaCl, and 1 mM TCEP. Fractions containing the protein of interest were pooled and concentrated such that the final concentration was in the range of 300-600 µM. 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 (GuHCl) and 25 mM EDTA. A sample of each protein was sent for electrospray ionization mass spectrometry (ESI-MS; Department of Chemistry, University of Toronto) and the determined molecular masses of the WT, H107A, and C142S proteins corresponded to their calculated molecular masses (Table 3-2). All proteins were > 90 % pure as estimated by Coomassie-stained SDS-PAGE that was analyzed by using the public domain NIH
The ImageJ program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/ij/).

**Preparation of proteins.** Reduced, apo-protein 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 (Amersham) equilibrated with protein buffer (25 mM HEPES, pH 7.6, and 100 mM KCl) in the glovebox. For experiments in buffer containing 100 mM NaCl, the columns were equilibrated with 25 mM HEPES, pH 7.6, and 100 mM NaCl. The absence of any metal bound to the protein was confirmed by a 4-(2-pyridylazo)resorcinol (PAR) assay, in which 10-20 µM protein was denatured with 4 M GuHCl and 50 μM PAR was added to the sample. The absorbance at 500 nm, due to the formation of the (PAR)\(_2\)Me(II) complex, was monitored and compared to a standard curve prepared with 50 μM PAR in 4 M GuHCl and known metal concentrations. The free thiol content of the proteins was measured via reaction of the protein with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in the presence of 6 M GuHCl 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 NiCl\(_2\) were prepared in the glovebox and incubated overnight at 4 °C. The electronic absorption spectrum was monitored between 250-500 nm and corrected by background subtraction at 600 nm. In samples containing nucleotide, 5 mM MgCl\(_2\) and 100 µM GDP or GDPNP were also included. Metal stoichiometry experiments were conducted by incubating 120 µM apo-*HpHypB* with either 600 µM NiCl\(_2\) or 500 µM ZnSO\(_4\) overnight at 4 °C in the glovebox. For samples containing additional ligands, 5 mM MgCl\(_2\), 500 µM GDP and/or 500 µM GDPNP were also included. Excess metal and ligand

### Table 3-2: Calculated and observed molecular masses (MM) for WT and mutant *HpHypB* as determined by ESI-MS.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Calculated MM (Da)</th>
<th>Observed MM (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT <em>HpHypB</em></td>
<td>27179.2</td>
<td>27179.0</td>
</tr>
<tr>
<td>C142S <em>HpHypB</em></td>
<td>27163.1</td>
<td>27163.0</td>
</tr>
<tr>
<td>H107A <em>HpHypB</em></td>
<td>27113.1</td>
<td>27113.0</td>
</tr>
</tbody>
</table>

*a*The values exclude the N-terminal methionine, which is removed.

ImageJ program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/ij/).
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 glovebox. The protein concentrations were calculated as described above. To confirm no interference from nucleotide, protein concentrations were verified with a BCA assay (Thermo Scientific), following the manufacturer’s instructions. The metal content was determined via a PAR assay.

**Magnesium and nucleotide-free WT *HpHypB* nickel affinity.** The nickel titration suggested that *HpHypB* binds nickel quantitatively under our buffer conditions, as previously reported. In order to determine the nickel affinity of WT *HpHypB*, the competitor Mag-fura-2 (MF2) was utilized as described previously. Stocks of MF2 (Invitrogen) were prepared in Milli-Q water and quantified by using the reported extinction coefficient of 22,000 M⁻¹ cm⁻¹ at 369 nm. To determine the metal-binding affinity of MF2 with Ni(II) under our experimental conditions, fluorescence spectroscopy was used. For determination of the Ni(II) *K_d*, 0 - 50 µM metal was added to individual samples of 5 nM MF2 in protein buffer and allowed to equilibrate for at least 2 h. MF2 was excited at 366 nm and the decreasing fluorescence upon metal complexation was monitored at 505 nm. The apparent *K_d* for MF2 was calculated by determining the fractional saturation, *r*, and free nickel concentration, [Ni(II)]ₜₚₑₑ, by using equations 1 and 2, respectively:

\[
r = \frac{[\text{MF-Ni(II)}]}{[\text{MF}]_{\text{total}}} = \frac{F_l_{\text{max}} - F_l_{\text{obs}}}{F_l_{\text{max}} - F_l_{\text{min}}} \quad (Eq. 1)
\]

\[
[Ni(II)]_{\text{free}} = [Ni(II)]_{\text{total}} - (r \times [MF]_{\text{total}}) \quad (Eq. 2)
\]

where [MF-Ni(II)] is the concentration of MF2 bound to Ni(II), [MF]ₜₚₑₑ is the total MF2 concentration, *F_l_{obs}* is the fluorescence intensity at 505 nm for a given Ni(II) concentration, *F_l_{max}* is the fluorescence at 505 nm for apo-HypB, *F_l_{min}* is the fluorescence at 505 nm upon saturation with Ni(II), and [Ni(II)]ₜₚₑₑ is the total Ni(II) concentration added to the sample. The resulting values were plotted as *r* vs. [Ni(II)]ₜₚₑₑ and the data fit in OriginPro 8 to the Hill equation (3):

\[
r = \frac{[Ni(II)]_{\text{free}}^n}{K_d^n + [Ni(II)]_{\text{free}}^n} \quad (Eq. 3)
\]
where $n$ is the Hill coefficient, which in this case was set to 1. The affinity of MF2 for nickel under our buffer conditions was determined to be $150 \pm 20$ nM, in agreement with the previously published value.\textsuperscript{9} 

Competition experiments were prepared by incubating 10 $\mu$M WT or mutant $Hp$HypB together with 10 $\mu$M MF2 and varying amounts of NiCl$_2$ in protein buffer. The samples were incubated overnight at 4 °C in the glovebox. The absorbance of MF2 at 366 nm was monitored using electronic absorption spectroscopy and the data were analyzed by using DYNAFIT\textsuperscript{37} employing a custom DYNAFIT script (Appendix II, pg. 191) that describes the competition between the protein and MF2 for the metal.

**WT $Hp$HypB Ni(II) affinity in the presence of magnesium and nucleotide.** The affinity of WT $Hp$HypB for Ni(II) in the presence of MgCl$_2$ 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) (Figure 3-10). 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 MgCl$_2$, 500 $\mu$M GDP or 500 $\mu$M GDPNP was determined by incubating 50 nM MF2 with the corresponding ligand and varying NiCl$_2$ concentrations for at least 2 hours. The decrease of the emission spectrum at 505 nm upon excitation at 335 nm was monitored via fluorescence and the data 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 $K_d$ was determined to be $(7 \pm 2) \times 10^{-7}$ M (Figure 3-10). Similarly, control titrations yielded apparent MF2 Ni(II) $K_d$s of 1.5 ± 0.3 $\mu$M and 1.8 ± 0.3 $\mu$M in the presence of GDPNP and GDP, respectively (Figure 3-10).

Competition experiments were prepared by incubating 10 $\mu$M WT apo-$Hp$HypB together with 5 $\mu$M MF2 and varying amounts of Ni(II) in the presence of 5 mM MgCl$_2$ and 500 $\mu$M GDP or GDPNP. The samples were incubated overnight at 4 °C in the glovebox and the fluorescence decrease at 505 nm upon excitation at 335 nm was monitored and the data were analyzed by using DYNAFIT\textsuperscript{37} employing a custom DYNAFIT script (Appendix II, pgs. 193 and 194) that describes the competition between the protein and MF2 for the metal. The equilibria used to fit the data included the protein dimerization step and the resulting $K_d$ possessed the units of $\mu$M$^2$. 
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) were incubated overnight at 4°C in the glovebox in protein buffer with increasing concentrations of NiCl₂. The electronic absorption spectrum was monitored between 250-500 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 $K_d$ was calculated by determining the fractional saturation, $r$, and free nickel concentration, $[\text{Ni(II)}]_{\text{free}}$, by using equations 4 and 5, respectively:

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

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

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 $[\text{Ni(II)}]_{\text{total}}$ is the total Ni(II) concentration added to the sample. The resulting values were plotted as $r$ vs. $[\text{Ni(II)}]_{\text{free}}$, and the data fit to the Hill equation (Eq. 3) as described above except that $n$ was allowed to vary.

*HpHypB* zincon competition for zinc. In order to estimate the Zn(II) $K_d$ of WT and mutant *HpHypB*, the competitor zincon was selected due to its ability to form a 1:1 complex with Zn(II) with a reported $K_d$ of 12.7 µM. 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 nM zincon in protein buffer with increasing amounts of ZnSO₄. The absorbance at 620 nm was monitored using a 10 cm pathlength cuvette and a GBC Cintra 404 spectrophotometer. The data were analyzed by using eqs. 4 and 5 and fit to the Hill equation (Eq. 3) with $n = 1$. Under our buffer conditions, the $K_d$ of zincon for Zn(II) was determined to be $7 \pm 1$ µM, in agreement with previously reported $K_d$s. Competition experiments were prepared by incubating 20 µM
apo-WT or mutant HpzHpB with 140 µM zinccon and various amounts of ZnSO₄. The samples were incubated overnight at 4 °C in the glovebox. The absorbance of zinccon at 620 nm was monitored using a 2 mm pathlength cuvette.

**WT HpzHpB 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 nM, in agreement with previously published values⁹,³⁶. The affinity of WT HpzHpB 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 previously described 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 (Figure 3-11A).⁴⁰ Thus, by monitoring the increasing fluorescence at 505 nm upon excitation at 321 nm while in the presence of 5 mM MgCl₂, 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 nM MF2 was used for the titrations, the fluorescence at 505 nm was monitored upon excitation 321 nm, and the fractional saturation was calculated by using equation 6:

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

where in this case \(F_{obs}\) is the fluorescence intensity at 505 nm for a given Zn(II) concentration, \(F_{max}\) is the fluorescence at 505 nm upon saturation of MF2 with Zn(II), and \(F_{min}\) is the fluorescence at 505 nm of apo MF2. In the presence of 5 mM Mg(II), the MF2 Zn(II) apparent \(K_d\) was determined to be \((2.7 ± 0.7) \times 10^{-7} \text{ M}\) (Figure 3-11). Similarly, control titrations yielded apparent MF2 Zn(II) \(K_d\)s of \((5.9 ± 0.9) \times 10^{-7} \text{ M}\) and \((3.8 ± 0.7) \times 10^{-7} \text{ M}\) in the presence of GDPNP and GDP, respectively (Figure 3-11).

Competition experiments were prepared by incubating 5 µM WT apo-HpzHpB together with 5 µM MF2 and varying amounts of Zn(II) in the presence of 5 mM MgCl₂ and 500 µM GDP or
GDPNP. The samples were incubated overnight at 4 °C in the glovebox and the decrease of the fluorescence at 505 nm upon excitation at 321 nm was monitored at 505 nm and the data were analyzed by using DYNAFIT\textsuperscript{37} employing a custom DYNAFIT script (Appendix II, pg. 195) that describes the competition between the protein and MF2 for the metal.

**Analytical gel filtration chromatography.** Samples containing 50 µM WT or mutant *Hp*HypB were incubated with the desired metal or nucleotide at various concentrations (see Figures 3-4 and 3-15) overnight at 4 °C in the glovebox. 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 chelexed 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), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B\(_{12}\) (1.4 kDa) from BioRad. No difference in the elution profile of these standard proteins was observed when 140 µM 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 (\(K_{av}\)), where \(K_{av} = (V_e - V_o)/(V_t - V_o)\); \(V_e\) represents the elution volume, \(V_o\) is the void volume, and \(V_t\) is the total column volume.

**Circular dichroism (CD) spectroscopy.** WT and mutant *Hp*HypB 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 µM and metal or nucleotide was added and incubated overnight at 4 °C in the glovebox (see Table 3-6 and Figures 3-6 and 3-7 for concentrations of added ligands). Protein samples with nucleotide included 5 mM MgCl\(_2\) and were also incubated overnight at 4 °C in the glovebox. All samples were analyzed on a Jasco J-170 spectropolarimeter with a capped 1 mm pathlength cuvette in order to minimize exposure to the air. Spectra were collected at 1 nm intervals over a spectral range of 200-260 nm with a scan speed of 20 nm min\(^{-1}\) at room temperature. The final spectra obtained are averages of six scans and corrected by subtracting the background buffer signal. The observed ellipticity was converted into mean residue ellipticity ([\(\theta\)]\textsubscript{mre}; deg cm\(^2\) dmol\(^{-1}\)) using the following formula:\textsuperscript{41}
\[
[\theta]_{mre} = \frac{\left(\frac{MW}{N-1}\right) \times \theta}{[Protein] \times l \times 10}
\]

where MW is the molecular weight 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 pathlength. The data were smoothed using a Savitzky-Golay function in OriginPro 8.

Thermal denaturation samples were prepared as described above. Spectra were collected at 1 nm intervals over a spectral range of 200-260 nm with a scan speed of 20 nm min\(^{-1}\) as the temperature was increased from 4 °C to 88 °C in 4 °C increments, with 1 min equilibration time between temperature increases. The signal at 222 nm was used to analyze the data and the observed ellipticity was converted into mean residue ellipticity as described above and plotted versus temperature in kelvin. There was a single clear transition in the thermal melts (Figure 3-6), so the data were fit in OriginPro 8 to the following equation, which describes a two-state transition:\(^{42}\)

\[
y = \left(\frac{U - L}{1 + e^{\frac{h}{1.987T_c} \times (\frac{\theta}{T_c} - 1)}}\right) + 1
\]

where \(U\) is the mean residue ellipticity of 100% folded helical protein, \(L\) is the mean residue ellipticity of unfolded protein, \(h\) is the starting enthalpy in cal mol\(^{-1}\) and \(T\) is the melting temperature of the protein (T\(_m\)) in kelvin.

**GTPase assays.** GTPase activity was determined by using the Malachite Green assay for free phosphate adapted from Lanzetta et al.\(^{43}\) A series of 400 µL samples containing 2 µM WT or H107A HpHypB (in 25 mM HEPES, pH 7.6, 100 mM NaCl or KCl), 5 mM MgCl\(_2\) and varying GTP concentrations were incubated at 37 °C in the glovebox for 30 min. Controls containing only buffer, 5 mM MgCl\(_2\) 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 and the color was allowed to develop for 30 min before plating on to a 96-well plate and the absorbance was measured at 630 nm with a Tecan 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 pre-incubated 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 the 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 with 500 µM NiCl₂ or 500 µM ZnSO₄ for H107A.

In order 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 (Figure 3-1). 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 pre-incubated 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 the 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 \( \text{HpHypB} \). To determine the affinity of WT and mutant \( \text{HpHypB} \) for GDP and GTP in the presence and absence of metal, 50 nM of fluorescently labeled GDP-BODIPY (guanosine 5'-diphosphate BODIPY Fl 2'-(or 3')-O-(N-2-aminoethyl) urethane), bis(triethylammonium) salt; Invitrogen) or GTP-BODIPY (guanosine 5'-triphosphate BODIPY Fl 2'-(or 3')-O-(N-2-aminoethyl) urethane), bis(triethylammonium) salt; Invitrogen) was incubated for at least 2 h at 4 °C in the dark in the glovebox with increasing amounts of protein. The change in the fluorescence of the BODIPY dyes excited at 488 nm was monitored at 509 nm. Protein samples containing metal were pre-incubated with either 500 µM NiCl\(_2\) or 200 µM ZnSO\(_4\) overnight in the glovebox prior to addition to the BODIPY-labeled nucleotides. All samples contained 25 mM HEPES, pH 7.6, 100 mM NaCl or KCl, and 5 mM MgCl\(_2\). The fluorescence intensity of 50 nM free-BODIPY-labeled nucleotide was subtracted prior to fitting the data to the Hill equation (Eq. 3).

Crystallization of \( \text{HpHypB} \) and X-ray Fluorescence Measurements. In order to avoid the formation of disulfide bonds between cysteine residues involved in nickel coordination, all the experiments were conducted under anaerobic conditions in a glove box. Prior to crystallization,
the protein was incubated for 2 h with 3 mM GTPγS and 3 mM MgCl2 at room temperature. A three-fold excess of NiCl2 was then added just before the crystallization step. Sitting drops were prepared using the AnoXtal platform (composed of a Gryphon robot and a Cryscam for drop visualization) by mixing 200 nL of a 20 mg/ml protein solution in 25 mM HEPES, pH 7.6, 100 mM NaCl, 2 mM TCEP with 200 nL of crystallization screens (Hampton Research Grid Screens™ and Qiagen protein crystallization suites). Among about 600 tested conditions, monoclinic crystals appeared either after a week in the presence of 1.0 M malonate, pH 7.0, or after several weeks in the presence of 4 M NaCl, 0.1 M Tris, pH 8.0. All the crystals were cryoprotected using a solution obtained by adding 15 % (v/v) glycerol to the reservoir solution and flash-cooled in liquid nitrogen. The X-ray fluorescence from HpHypB crystals was monitored with a solid state Röntec XFlash detector operating at the BM30A beamline at the European Synchrotron Radiation Facility (ESRF), corroborating the presence of nickel in the crystals.

Data Collection and Structure Determination. Data were collected at beamline BM30A of the ESRF in Grenoble, France. Data reduction was carried out using XDS. Crystallographic statistics are summarized in Table 3-3. In order 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: $\lambda_{\text{Sc}} = 0.97969 \, \text{Å}$ (maximum $f''$ for selenium), $\lambda_{\text{Zn1}} = 1.28212$, $\lambda_{\text{Zn2}} = 1.28616$, $\lambda_{\text{Ni1}} = 1.48627 \, \text{Å}$, $\lambda_{\text{Ni2}} = 1.49162 \, \text{Å}$ (corresponding to both sides of maximum $f''$ for zinc and nickel, respectively). The structure was solved with PHASER by the molecular replacement method using as a starting model the atomic coordinates of the HypB X-ray model from Methanocaldococcus jannaschii (PDB ID code 2HF9). Crystallographic refinements were conducted using PHENIX and the three-dimensional models were examined and modified using the graphics program COOT. Superimpositions of models and root mean square deviations (rmsd) were calculated using the Secondary-Structure Matching (SSM) tool. Figures 3-12 and 3-13 were prepared with PyMOL (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC.).
Table 3-3. Data collection and refinement statistics for data sets used to solve the HpHypB structure and to determine the presence of nickel at the binding site.

<table>
<thead>
<tr>
<th></th>
<th>HypB at ( \lambda_{\text{Se}} )</th>
<th>HypB at ( \lambda_{\text{Zn1}}^* )</th>
<th>HypB ( \lambda_{\text{Zn2}}^* )</th>
<th>HypB at ( \lambda_{\text{Ni1}}^* )</th>
<th>HypB at ( \lambda_{\text{Ni2}}^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beamlines</td>
<td>BM30A</td>
<td>BM30A</td>
<td>BM30A</td>
<td>BM30A</td>
<td>BM30A</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>0.97969</td>
<td>1.28212</td>
<td>1.28616</td>
<td>1.48627</td>
<td>1.49162</td>
</tr>
<tr>
<td>Unit cell</td>
<td>( a = 49.82 \text{ Å}; b = 50.16 \text{ Å}; c = 53.68 \text{ Å}; \alpha = 102.155^\circ; \beta = 99.050^\circ; \gamma = 90.649^\circ )</td>
<td>( a = 49.87 \text{ Å}; b = 50.20 \text{ Å}; c = 53.73 \text{ Å}; \alpha = 102.135^\circ; \beta = 99.068^\circ; \gamma = 90.652^\circ )</td>
<td>( a = 49.92 \text{ Å}; b = 50.26 \text{ Å}; c = 53.84 \text{ Å}; \alpha = 102.121^\circ; \beta = 99.125^\circ; \gamma = 90.710^\circ )</td>
<td>( a = 49.96 \text{ Å}; b = 50.32 \text{ Å}; c = 53.97 \text{ Å}; \alpha = 102.099^\circ; \beta = 99.133^\circ; \gamma = 90.795^\circ )</td>
<td>( a = 50.01 \text{ Å}; b = 50.39 \text{ Å}; c = 54.12 \text{ Å}; \alpha = 102.045^\circ; \beta = 99.174^\circ; \gamma = 90.892^\circ )</td>
</tr>
<tr>
<td>Space group</td>
<td>P1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of molecules per asymmetric units</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>40.15 - 2.00</td>
<td>35.56 - 2.10</td>
<td>40.240 - 2.20</td>
<td>33.974 - 2.55</td>
<td>33.985 - 2.55</td>
</tr>
<tr>
<td>[2.10 - 2.00]</td>
<td>[2.20 - 2.10]</td>
<td>[2.30 - 2.20]</td>
<td>[2.65 - 2.55]</td>
<td>[2.65 - 2.55]</td>
<td>[2.65 - 2.55]</td>
</tr>
<tr>
<td>( R_{\text{sym}} ) (%)</td>
<td>9.9 [51.6]</td>
<td>5.9 [30.0]</td>
<td>6.2 [32.0]</td>
<td>8.1 [42.8]</td>
<td>5.6 [38.6]</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>97.7 [96.5]</td>
<td>92.4 [88.8]</td>
<td>93.4 [92.7]</td>
<td>93.7 [91.8]</td>
<td>93.5 [92.6]</td>
</tr>
<tr>
<td>( N_{\text{measured}} )</td>
<td>128517 [17168]</td>
<td>106621 [13149]</td>
<td>94365 [11742]</td>
<td>61048 [6668]</td>
<td>61089 [6681]</td>
</tr>
</tbody>
</table>

Refinement statistics

| R factor/ \( R_{\text{free}} \) factor (%) | 16.9 / 19.9 |
| rmsd - Bonds (Å) | 0.007 |
| rmsd - Angles (°) | 1.23 |
| Ramachandran plot | 98.6 |
| Residues in most favorable region (%) | 0.2 |
| Residues in disallowed region (%) | * Friedel pairs are treated as different reflections |
3.3 Results

**HpHypB is a potassium-activated enzyme.** A recent study of the [FeFe]-hydrogenase maturation factor HydF, which is also a GTPase, demonstrated that GTP hydrolysis is faster in the presence of potassium,\(^9\) prompting us to investigate the impact of potassium on the GTPase activity of *HpHypB*. In the presence of 100 mM KCl, the \(k_{cat}/K_m\) of the enzyme increased to 120 ± 38 M\(^{-1}\) s\(^{-1}\) (Table 3-4 and Figure 3-2) from the previously determined \(k_{cat}/K_m\) of 12 ± 3 M\(^{-1}\) s\(^{-1}\) in 100 mM NaCl.\(^9\) This change is solely due to an increase in \(k_{cat}\) (Table 3-4 and Figure 3-3), suggesting that potassium plays a role in the rate-limiting step of the enzyme. Given that the concentration of potassium used in the GTPase assays is in the range estimated for *H. pylori* cytoplasmic conditions\(^50\) and that sodium did not appear to have any impact on activity (Figure 3-2), potassium was included in all subsequent solution experiments. Previous analysis demonstrated that GTP hydrolysis by HypB in NaCl is modified by nickel and zinc,\(^9,51,52\) so experiments were performed to determine if this impact of metal on *HpHypB* is maintained in the presence of KCl. The GTPase activity of the Ni(II)-loaded enzyme is weakened compared to apo-protein, resulting in a diminished \(k_{cat}\) and \(K_m\) and a reduction in the overall catalytic efficiency by more than 30-fold to 3.5 ± 0.2 M\(^{-1}\) s\(^{-1}\) (Table 3-4). In the case of Zn(II), low levels of GTPase activity were detected but they were too weak to calculate kinetic constants (Figure 3-2).

Given the impact of potassium on the enzymatic activity of *HpHypB*, the other biochemical properties of the protein were re-analyzed. In analytical gel filtration experiments the apo-protein still eluted as a monomer and only nickel, and not zinc or nucleotide, induced dimer formation (Figure 3-4). Competition experiments between *HpHypB* and the metal chelator Magfura-2 (MF2) revealed an apparent \(K_d\) of the protein for nickel of 210 ± 70 nM (Table 3-5 and Figure 3-5), similar to the \(K_d\) of 150 nM determined in NaCl.\(^9\) The impact on zinc binding was greater, with the apparent \(K_d\) increasing from 1.2 nM in NaCl\(^9\) to 57 ± 5 nM in KCl (Figure 3-5).
Impact of GDP and Zn(II) on the melting temperature of HpHypB. To elucidate the full impact of potassium on HpHypB, thermal melts of apo-HpHypB as well as the protein bound to Zn(II), Ni(II), GDP, the GTP analog guanosine 5'-[β,γ-imido]triphosphate (GDPNP), and Na⁺ were monitored by circular dichroism spectroscopy (Figure 3-6). The CD spectrum of the protein is typical of a mixed αβ protein, in accordance with the determined crystal structure, and was unaltered by the addition of NaCl, Ni(II), Zn(II), GDP, or GDPNP (Figure 3-7). Upon monitoring the thermal denaturation of the protein at 222 nm, a single unfolding transition was evident under all conditions tested (Figure 3-6). The apo-WT protein in potassium phosphate buffer has a Tₘ of 41 ± 1 °C, which is not significantly different from the Tₘ of the protein in

**Table 3-4. Kinetics of GTP hydrolysis by WT, H107A, and C142S HpHypB.**

<table>
<thead>
<tr>
<th>HpHypB Variant</th>
<th>Condition</th>
<th>kₘₐₓ (s⁻¹)</th>
<th>Kₘ (M)</th>
<th>kₘₐₓ/Kₘ (M⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Apo in 100 mM NaCl</td>
<td>(6 ± 1) x 10⁻⁴</td>
<td>(5 ± 1) x 10⁻⁵</td>
<td>12 ± 3</td>
<td></td>
</tr>
<tr>
<td>Apo in 100 mM KCl</td>
<td>(6 ± 1) x 10⁻³</td>
<td>(5 ± 2) x 10⁻⁵</td>
<td>120 ± 38</td>
<td></td>
</tr>
<tr>
<td>+ 200 µM NiCl₂</td>
<td>(2.2 ± 0.4) x 10⁻³</td>
<td>(6 ± 1) x 10⁻⁴</td>
<td>3.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>+ 100 µM ZnSO₄</td>
<td>(6 ± 1) x 10⁻³</td>
<td>(5.2 ± 0.8) x 10⁻⁵</td>
<td>113 ± 19</td>
<td></td>
</tr>
<tr>
<td>+ 100 mM NaCl</td>
<td>(6 ± 1) x 10⁻³</td>
<td>(5.2 ± 0.8) x 10⁻⁵</td>
<td>113 ± 19</td>
<td></td>
</tr>
<tr>
<td>Apo in 100 mM KCl, 30°C</td>
<td>(3.6 ± 0.6) x 10⁻³</td>
<td>(4.9 ± 0.7) x 10⁻⁵</td>
<td>76 ± 19</td>
<td></td>
</tr>
<tr>
<td>H107A Apo</td>
<td>(8 ± 1) x 10⁻³</td>
<td>(1.3 ± 0.2) x 10⁻⁴</td>
<td>62 ± 11</td>
<td></td>
</tr>
<tr>
<td>+ 500 µM NiCl₂</td>
<td>(1.7 ± 0.7) x 10⁻³</td>
<td>(1.3 ± 0.3) x 10⁻⁴</td>
<td>12 ± 3</td>
<td></td>
</tr>
<tr>
<td>+ 500 µM ZnSO₄</td>
<td>(1.4 ± 0.3) x 10⁻³</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>C142S Apo</td>
<td>(1.4 ± 0.3) x 10⁻³</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>+ 1 mM NiCl₂</td>
<td>(8 ± 2) x 10⁻⁴</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>+ 500 µM ZnSO₄</td>
<td>(7 ± 2) x 10⁻⁴</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

*aAll GTPase assays were conducted with 2 µM WT, H107A, or C142S HpHypB in protein buffer containing 100 mM KCl supplemented with 5 mM MgCl₂. The proteins were preincubated with the indicated amounts of nickel or zinc overnight at 4 °C in an anaerobic glove box. The data listed are average values from at least three independent experiments. ND, not determined; *bPreviously published.*

Impact of GDP and Zn(II) on the melting temperature of HpHypB. To elucidate the full impact of potassium on HpHypB, thermal melts of apo-HpHypB as well as the protein bound to Zn(II), Ni(II), GDP, the GTP analog guanosine 5'-[β,γ-imido]triphosphate (GDPNP), and Na⁺ were monitored by circular dichroism spectroscopy (Figure 3-6). The CD spectrum of the protein is typical of a mixed αβ protein, in accordance with the determined crystal structure, and was unaltered by the addition of NaCl, Ni(II), Zn(II), GDP, or GDPNP (Figure 3-7). Upon monitoring the thermal denaturation of the protein at 222 nm, a single unfolding transition was evident under all conditions tested (Figure 3-6). The apo-WT protein in potassium phosphate buffer has a Tₘ of 41 ± 1 °C, which is not significantly different from the Tₘ of the protein in
sodium phosphate (Table 3-6). This $T_m$ is surprisingly low considering that *H. pylori* is exposed to temperatures of 37 °C in the human digestive tract. Furthermore, the GTPase assays were conducted at 37 °C, a temperature at which a portion of enzyme may be unfolded based on this $T_m$. However, GTPase assays conducted at 30 °C yielded lower enzymatic activity compared to assays conducted at 37 °C (Table 3-4). Furthermore, when the GTP analog was added to *Hp*HypB, the $T_m$ increased to 45 ± 2 °C (Table 3-6). A more drastic change was observed in the GDP-bound protein, as the $T_m$ increased to 52 ± 2 °C. The addition of Ni(II) did not drastically change the melting temperature, but a significant increase ($p < 0.05$) was observed when Zn(II) was added (Table 3-6).

**Figure 3-2. GTPase activity of HpHypB.** Apo-*Hp*HypB (2 µM, circles) was incubated in buffer containing 100 mM KCl with increasing concentrations of GTP at 37 °C for 30 min in an anaerobic glovebox prior to detection of released inorganic phosphate using a colorimetric Malachite green assay. Fitting the data from several experiments to the Michaelis-Menten equation (solid black line) yields a $k_{cat}/K_m$ of 120 ± 38 M$^{-1}$s$^{-1}$. The addition of 100 mM NaCl (squares) did not change the activity of the enzyme (short dotted line, fit; $k_{cat}/K_m = 113 ± 19$ M$^{-1}$ s$^{-1}$). The presence of 200 µM NiCl$_2$ (triangles) lowered the GTPase activity of the enzyme, resulting in a $k_{cat}/K_m$ of 3.5 ± 0.2 M$^{-1}$ s$^{-1}$ (long dashed line, fit). Similarly, the addition of 100 µM ZnSO$_4$ (X) also impaired the enzyme, but to a greater degree. In the case of zinc, saturation was not achieved (solid grey line, linear fit), suggesting a significantly diminished $K_m$. The full range of GTP concentrations used is depicted in the graph on the left, with an enlargement of the 0-1000 µM range on the right. All datasets are the average of at least three independent replicates and error bars represent the standard deviations of the replicates.
Figure 3-3. Dependence of $k_{cat}$ on [KCl]. Apo-WT HpHypB (2 µM, squares) was incubated with 950 µM GTP for 20-30 min at 37 °C in an anaerobic glovebox with increasing amounts of KCl prior to detection of released inorganic phosphate by a malachite green colorimetric assay. The $k_{cat}$ of the enzyme gradually increases as the concentration of KCl in the buffer increases. The dataset is the average of four independent replicates and error bars represent the standard deviation.

Figure 3-4. Effect of metal and nucleotide on the quaternary structure of WT HpHypB. (A) In the absence of metal, 50 µM WT HpHypB elutes at a volume corresponding to a monomer (solid black line). The addition of 120 µM NiCl$_2$ (dashed line) results in the formation of a dimeric species. The addition of 120 µM ZnSO$_4$ (grey dotted line) or 500 µM GDP and 250 µM ZnSO$_4$ (solid grey line) does not alter the quaternary structure of HpHypB and the protein still elutes as a monomer. (B) Incubation of WT HpHypB with 2 mM GDP (grey dotted line) or the GTP analog guanosine 5’-[β,γ-imido]triphosphate (dashed line) does not change the oligomeric state of the protein. The large peak at 20-25 mL is due to free nucleotide. The chromatographic traces (monitored at 280 nm) are representative data sets from experiments with a Superdex 200 10/300 analytical column equilibrated with 25 mM HEPES, pH 7.6, 200 mM KCl, and 5 mM MgCl$_2$. The ticks at the top of the graph denote the elution volumes of the protein standards. From left to right, the identities of the standards (and molecular masses) are thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B$_{12}$ (1.4 kDa), respectively.
Figure 3-5. Affinity of HpHypB for Ni(II) and Zn(II) in potassium-containing buffer. In order to measure the strength of the HpHypB-Ni(II) or HpHypB-Zn(II) interactions, a competition between 5 µM apo-WT HpHypB and 10 µM of the metal chelator Mag-fura-2 (MF2) for Ni(II) (circles; fit, solid line) or Zn(II) (squares; fit, dashed line) was established. By monitoring the decrease of the apo MF2 absorption at 366 nm upon metal binding as a function of added metal, the HpHypB Ni(II) and Zn(II) $K_d$s were determined to be $210 \pm 70$ nM and $57 \pm 5$ nM, respectively, using a custom script in DynaFit. The HpHypB-MF2 Ni(II) competition saturates at a higher absorbance than the Zn(II) competition due to a small contribution from the protein’s Cys$\rightarrow$Ni(II) LMCT. Control experiments correcting for this contribution demonstrated that it does not affect the results of the competition (data not shown). Representative data sets are shown.
Table 3-5. Apparent Ni(II) $K_d$ for WT and mutant $Hp$HypB with or without nucleotide.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Additional Ligands</th>
<th>Apparent $K_d$ (µM)</th>
<th>n</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT</strong></td>
<td>Mg(II)</td>
<td>320 ± 40 nM</td>
<td>N/A</td>
<td>MF2 Competition$^a$</td>
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<tr>
<td></td>
<td>GDPNP</td>
<td>0.4 ± 0.1 µM$^2$</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GDP</td>
<td>5 ± 2 µM$^2$</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- - -</td>
<td>170 ± 50 µM</td>
<td>1.7 ± 0.3</td>
<td></td>
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<tr>
<td><strong>C142S</strong></td>
<td>GDPNP</td>
<td>300 ± 100 µM</td>
<td>1.8 ± 0.5</td>
<td>Ni(II) Titration$^b$</td>
</tr>
<tr>
<td></td>
<td>GDP</td>
<td>No detectable binding</td>
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<tr>
<td></td>
<td>- - -</td>
<td>2.89 ± 0.04 mM</td>
<td>1.3 ± 0.1</td>
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<tr>
<td><strong>H107A</strong></td>
<td>GDPNP</td>
<td>&lt; 10 µM$^c$</td>
<td>N/A</td>
<td>Ni(II) Titration$^b$</td>
</tr>
<tr>
<td></td>
<td>GDP</td>
<td>50 ± 10 µM</td>
<td>1.0 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

$^a$To determine the affinity of WT $Hp$HypB for nickel, a competition between 10 µM WT $Hp$HypB 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 other WT samples, fluorescence spectroscopy was used. In both cases, the resulting data were fit in DynaFit. The equilibria used to fit the data from samples containing nucleotide included the protein dimerization step and the resulting $K_d$ possessed the units of M$^2$.

$^b$For nickel binding to apo-C142S and apo-H107A $Hp$HypB, 5 µM (for C142S + GDP and H107A + GDP) or 10 µM mutant protein (for all other nickel titration) were incubated overnight at 4 °C in the glovebox in protein buffer with increasing concentrations of NiCl$_2$, followed by electronic absorption spectroscopy. In samples containing nucleotide, 5 mM MgCl$_2$ and 500 µM GDP or GDPNP were also included. The data were fit to the Hill equation as described in the Experimental.

$^c$A Ni(II) titration of 10 µM H107A in the presence of GDPNP revealed binding curves similar to that of WT protein + GDPNP, so Ni(II) binding was too tight to extract a $K_d$ and an upper limit of 10 µM was estimated.
Figure 3-6. Thermal denaturation curves of WT *Hp*HypB monitored by circular dichroism spectroscopy. The denaturation of 20 µM protein was monitored on an Olis RSM 1000 spectropolarimeter with a capped 1 mm pathlength cuvette at 222 nm as the temperature was increased from 4 °C to 88 °C. The data were analyzed as described in the Materials and Methods. (A) WT *Hp*HypB, squares, fit- solid black line; WT + 100 µM Ni(II), triangles, fit- dashed black line; WT + 100 µM Zn(II), X, fit- dashed grey line; (B) WT *Hp*HypB, squares, fit- solid black line; WT + 500 µM GDP, triangles, fit- solid grey line; WT + 500 µM guanosine 5′-[β,γ-imido]triphosphate, circles, fit-black dashed line. The datasets shown are representative data sets. For clarity, the C142S and WT + Na⁺ datasets were excluded from the graph as they yielded nearly identical curves as apo WT.

### Table 3-6. Melting temperatures of WT and C142S *Hp*HypB determined by circular dichroism spectroscopy.a

<table>
<thead>
<tr>
<th><em>Hp</em>HypB Variant</th>
<th>Ligand</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Apo (in K⁺ buffer)</td>
<td>42 ± 1</td>
</tr>
<tr>
<td></td>
<td>Apo (in Na⁺ buffer)</td>
<td>40 ± 1</td>
</tr>
<tr>
<td>WT</td>
<td>100 µM Ni(II)</td>
<td>44 ± 1</td>
</tr>
<tr>
<td></td>
<td>100 µM Zn(II)</td>
<td>46 ± 2 *</td>
</tr>
<tr>
<td></td>
<td>500 µM GDP</td>
<td>52 ± 2 **</td>
</tr>
<tr>
<td></td>
<td>500 µM GTP analog</td>
<td>45 ± 2</td>
</tr>
<tr>
<td>C142S</td>
<td>Apo (in K⁺ buffer)</td>
<td>40 ± 1</td>
</tr>
</tbody>
</table>

*aAll thermal melts were conducted with 20 µM WT or C142S *Hp*HypB in 100 mM potassium phosphate buffer, pH 7.6, with the exception of the WT *Hp*HypB sample in Na⁺ buffer (100 mM sodium phosphate buffer, pH 7.6). Denaturation of the protein was monitored on an Olis RSM 1000 spectropolarimeter with a capped 1 mm pathlength cuvette at 222 nm as the temperature was increased from 4 °C to 88 °C. The data were analyzed as described in the Materials and Methods. The GTP analog used was guanosine 5′-[β,γ-imido]triphosphate. The data listed are average values from at least three independent experiments. *- indicates data are significantly different from that of apo-WT in K⁺ buffer, p < 0.05; **- indicates data are significantly different from that of apo-WT in K⁺ buffer, p < 0.01.
Figure 3-7. Circular dichroism (CD) spectra of WT, H107A, and C142S HpHypB. (A) The CD spectrum of 20 μM apo-WT HpHypB in KCl (black circles) possesses the general characteristics consistent with a mixed αβ protein, in agreement with the crystal structure. The structure in KCl is similar to the structure in NaCl (green circles), suggesting that the salt does not impact the protein’s overall structure. Upon the addition of 100 μM NiCl₂ (red squares) or 100 μM ZnSO₄ (blue triangles), no major structural changes were observed, except for a slight decrease in the intensity of the absorbance. (B) The addition of 500 μM GDP (red squares) or 500 μM GDPNP (blue triangles) did not drastically alter the secondary structure of the protein compared to apo-WT HpHypB (black circles). (C) The CD spectrum of 20 μM apo-H107A HpHypB (green circles) is similar to that of apo-WT HpHypB (black circles). The addition of 500 μM NiCl₂ (red squares) or 500 μM ZnSO₄ (blue triangles) did not alter the CD spectrum of H107A HpHypB. (D) The CD spectrum of 10 μM apo-C142S HpHypB (green circles) shows no deviations from that of apo-WT HpHypB (black circles). The addition of 500 μM NiCl₂ (red squares) or 500 μM ZnSO₄ (blue triangles) did not drastically alter the CD spectrum of C142S HpHypB.
Zinc, but not nickel, alters the nucleotide affinity of \textit{HpHypB}. Binding studies were conducted with BODIPY-labeled nucleotides to determine if potassium has any impact on the affinity of \textit{HpHypB} for GDP or the GTP, but no significant difference was observed in NaCl- vs. KCl-containing buffers (Table 3-7 and Figure 3-8). Inclusion of Ni(II) also had no effect on nucleotide affinity (Table 3-7), a somewhat surprising result given that the GTPase assays indicated that the $K_m$ of the protein increases by nearly an order of magnitude in the presence of Ni(II) (Table 3-4). In contrast, zinc weakened the protein’s ability to bind both GTP and GDP (Table 3-7). This decreased nucleotide binding ability may contribute to the severely reduced GTP hydrolysis by \textit{HpHypB} upon zinc loading (Figure 3-2).

**Table 3-7.** Affinity of nucleotide binding to WT, H107A, and C142S \textit{HpHypB}.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Nucleotide</th>
<th>Metal</th>
<th>$K_d$ (µM)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>GDP</td>
<td>---</td>
<td>1.4 ± 0.3</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>In NaCl</td>
<td>1.9 ± 0.7</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ni(II)</td>
<td>1.8 ± 0.6</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Zn(II)</td>
<td>14 ± 3</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>GTP</td>
<td>---</td>
<td>2.7 ± 0.3</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>In NaCl</td>
<td>3.2 ± 0.7</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ni(II)</td>
<td>3 ± 1</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Zn(II)</td>
<td>6 ± 1</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>C142S</td>
<td>GDP</td>
<td>---</td>
<td>1.7 ± 0.4</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>GTP</td>
<td>---</td>
<td>9 ± 2</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>H107A</td>
<td>GDP</td>
<td>---</td>
<td>1.6 ± 0.5</td>
<td>1.09 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>GTP</td>
<td>---</td>
<td>5 ± 2</td>
<td>1.1 ± 0.1</td>
</tr>
</tbody>
</table>

<sup>a</sup>To determine the affinity of WT and mutant \textit{HpHypB} for nucleotides in the presence and absence of metal, 50 nM of fluorescently labeled GDP-BODIPY or GTP-BODIPY was incubated for at least 2 hours at 4 °C in the dark with increasing amounts of protein. The fluorescence of the BODIPY dyes excited at 488 nm was monitored at 509 nm. Protein samples containing metal were pre-incubated with either 500 µM NiCl$_2$ or 200 µM ZnSO$_4$ overnight in the glovebox prior to addition of the BODIPY-labeled nucleotides. All samples contained 25 mM HEPES, pH 7.6, 100 mM NaCl or KCl, and 5 mM MgCl$_2$. The fluorescence intensity of 50 nM free-BODIPY F1 nucleotide was subtracted prior to fitting the data to the Hill equation.
Figure 3-8. Nucleotide binding to WT and mutant H\text{p}HypB. BODIPY-labelled GDP and GTP were used to determine the binding constants to WT and mutant H\text{p}HypB in the presence or absence of various metals. (A) BODIPY-GDP binding to WT H\text{p}HypB in the presence of 100 mM KCl (black circles; fit, black solid line), 100 mM NaCl (grey circles; fit, grey solid line), 500 µM NiCl\textsubscript{2} (black squares; fit, dotted line), 200 µM ZnSO\textsubscript{4} (triangles; fit, dashed line). (B) BODIPY-GTP binding to WT H\text{p}HypB in the presence of 100 mM KCl (black circles; fit, black solid line), 100 mM NaCl (grey circles; fit, grey solid line), 500 µM NiCl\textsubscript{2} (black squares; fit, dotted line), 200 µM ZnSO\textsubscript{4} (triangles; fit, dashed line). (C) BODIPY-GDP binding to H107A H\text{p}HypB (grey triangles; fit, grey solid line) and C142S H\text{p}HypB (black circles; fit, solid black line). BODIPY-GTP binding to H107A (grey circles; fit, grey dashed line) and C142S H\text{p}HypB (squares; fit, dashed black line). Representative datasets are shown fit to the Hill equation.
Nucleotide modulates nickel binding to *HpHypB*. Given the inhibition of GTP hydrolysis by transition metal binding to *HpHypB*, it is reasonable to expect that a link exists between the nucleotide-bound state of *HpHypB* and metal coordination. This model is consistent with the previously reported observation that GTP binding alters the ligand-to-metal charge transfer (LMCT) bands in the electronic absorption spectrum of the Ni(II)-loaded protein. To explore this effect further, 20 µM protein was incubated with nickel in the presence or absence of 100 µM GDP or GDPNP. In the absence of nucleotide, a Cys → Ni(II) LMCT peak at 350 nm was observed (Figure 3-9A), as previously reported. The addition of GDP or GDPNP shifted the peak to 330 and 337 nm, respectively, suggesting a change in the Ni(II) coordination sphere (Figure 3-9B). Furthermore, the Ni(II)-protein signal in the presence of nucleotide saturated with 0.5 equivalents of metal, as opposed to one equivalent in the absence of nucleotide (Figure 3-9B). 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 3-8). In contrast, the zinc stoichiometry remained unaltered, regardless of the nucleotide-bound state of *HpHypB* (Table 3-8).

MF2 competition experiments were used to measure the affinity of WT *HpHypB* for nickel in the presence of nucleotide, as described in the Materials and Methods. The WT *HpHypB* apparent Ni(II) *Kd* in the presence of 5 mM Mg(II) was determined to be 320 ± 40 nM, in close agreement with the Ni(II) *Kd* determined in the absence of Mg(II) (Table 3-5 and Figure 3-10). In the presence of 500 µM GDPNP and 5 mM MgCl₂, the apparent *Kd* for Ni(II) was 0.4 ± 0.10 µM² (Table 3-5 and Figure 3-10). In contrast, the inclusion of 500 µM GDP and 5 mM MgCl₂, yields a weaker apparent *Kd* of 5 ± 2 µM² (Table 3-5 and Figure 3-10). Competitions with MF2 were also conducted to measure the affinity of WT *HpHypB* for zinc in the presence of nucleotide (Figure 3-11). The affinity of *HpHypB* for Zn(II) in the presence of Mg(II), GDP, and GDPNP were calculated to be 40 ± 20 nM, 48 ± 6 nM, and 120 ± 20 nM, respectively.
Figure 3-9. Nickel binding to WT HpHypB in the presence of GDP and GDPNP. (A) The difference spectra of 20 µM HpHypB incubated with 20 µM NiCl₂ (black solid line) displays a ligand-to-metal charge transfer (LMCT) band centered around 350 nm. This band is unchanged in the presence of 5 mM MgCl₂ (black dashed line), but shifts to 337 nm and 330 nm when either the GTP analog guanosine 5′-[β,γ-imido]triphosphate (GDPNP; solid grey line) or GDP are added (dashed grey line). (B) Upon titration of 20 µM apo-HpHypB with NiCl₂, a linear increase in the absorbance at 350 nm is observed until approximately 1 equivalent of metal has been added (black squares). The same trend is observed in the presence of 5 mM MgCl₂ (green squares). The addition of either GDP (blue squares) or GDPNP (red squares) causes the absorbance at 330 and 337 nm, respectively, to saturate at half an equivalent of added nickel. The extinction coefficients were calculated on the basis of the total protein concentration in each sample. Lines have been included to illustrate the trend and highlight the saturation point.

Table 3-8. Stoichiometry of metal binding to WT HpHypB.\(^a\)

<table>
<thead>
<tr>
<th>Metal Added</th>
<th>Additional Ligands</th>
<th>Metal Bound(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni(II)</td>
<td>- - -</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>5 mM MgCl₂</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>500 µM GDP</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>500 µM GDPNP</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Zn(II)</td>
<td>- - -</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>5 mM MgCl₂</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>500 µM GDP</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>500 µM GDPNP</td>
<td>0.9 ± 0.2</td>
</tr>
</tbody>
</table>

\(^a\)Apo-HpHypB (120 µM) was incubated with 600 µM of either Ni(II) or Zn(II) in the presence or absence of additional ligands overnight at 4 °C in an anaerobic glovebox. All samples containing nucleotide also contained 5 mM MgCl₂. Excess metal and ligand was removed by passing the proteins over a gel filtration column, and metal was detected via a PAR assay.

\(^b\)The data listed are average values and standard deviations of the number of metal ions per protein monomer from at least three independent experiments.
Figure 3-10. Nickel affinity of WT HpHypB in the presence of nucleotide. (A) Excitation spectrum of MF2 monitored at 505 nm. In the presence of Mg(II), Mag-fura-2 (MF2) fluoresces at 505 nm, with an excitation maximum at 335 nm. As nickel is added to the sample, Ni(II) can outcompete Mg(II) for MF2 and causes a decrease in the 505 nm fluorescence when excited at 335 nm. This differential response between Ni(II) and Mg(II) allows for the monitoring of Ni(II) binding in the presence of Mg(II). (B-D) By monitoring the decrease of the fluorescence of MF2 at 505 nm ($\lambda_{ex} = 335$ nm) upon Ni(II) binding and fitting these data versus the amount of Ni(II) added using a custom DynaFit script, the $HpHypB$ apparent Ni(II) $K_d$ was determined to be $320 \pm 40$ nM in the presence of Mg(II) (B; squares; solid line, fit). Similarly, in the presence of GDPNP and MgCl$_2$, the apparent $K_d$ for Ni(II) was found to be $0.4 \pm 0.1$ µM$^2$ (C; triangles; dashed line, fit). The addition of GDP and MgCl$_2$, weakened the affinity of $HpHypB$ for Ni(II), resulting in an apparent $K_d$ of $5 \pm 2$ µM$^2$ (D; circles; dotted line, fit). Insets in parts B-D are 5 nM MF2 titrations used to determine the apparent $K_d$ of MF2 in the presence of MgCl$_2$ [B; $K_{d(seq)} = (7 \pm 2) \times 10^{-7}$ M], GDP and MgCl$_2$ [C; $K_{d(seq)} = 1.8 \pm 0.3$ µM], and GDPNP and MgCl$_2$ [D; $K_{d(seq)} = 1.5 \pm 0.3$ µM]. Protein samples contained 10 µM $HpHypB$, 5 µM MF2, 5 mM MgCl$_2$ and 500 µM nucleotide. The squares (B-D) are 5 µM MF2 titrated with NiCl$_2$ for comparison.
Figure 3-11. Zinc affinity of WT HpHypB in the presence of nucleotide. (A) The excitation spectrum of MF2 in the presence of MgCl₂, monitored at 505 nm, features an excitation maximum at 335 nm (black line). As zinc is added and displaces the Mg(II) bound to MF2, the excitation spectrum at higher wavelengths decreases with a simultaneous increase at lower wavelengths, resulting in an isosbestic point at 343 nm. The excitation peak at 321 nm can be used to monitor Zn(II) binding to MF2 in the presence of Mg(II). (B) By monitoring the fluorescence at 505 nm (λ<sub>ex</sub> = 321 nm), the apparent Zn(II) K<sub>d</sub> of MF2 can be calculated in the presence of Mg(II) (black diamonds; fit, black line, apparent K<sub>d</sub> = (2.7 ± 0.7) x 10<sup>-7</sup> M), GDP (red squares; fit, red line, apparent K<sub>d</sub> = (3.8 ± 0.7) x 10<sup>-7</sup> M), and GDPNP (blue triangles; fit, blue line, apparent K<sub>d</sub> = (5.9 ± 0.9) x 10<sup>-7</sup> M). (C) In the absence of protein, 5 µM MF2 titrated with Zn(II) features a steep increase in fluorescence and saturates around 10 µM added Zn(II) in the presence of Mg(II) and/or nucleotide (black circles). Addition of 5 µM WT HpHypB to the sample allows for Zn(II) competition and by fitting the data using a custom DynaFit script, the apparent K<sub>d</sub> can be calculated to be 40 ± 20 nM in the presence of 5 mM Mg(II) (black diamonds; fit, black line), 48 ± 6 nM in the presence of 500 µM GDP and 5 mM MgCl₂ (red squares; fit, red line), and 120 ± 20 nM in the presence of 500 µM GDPNP and 5 mM MgCl₂ (blue triangles; fit, blue line). Representative datasets are shown below.
Crystal structure of Ni(II)-loaded HpHypB. To examine the structure of HpHypB bound to nickel, the crystal structure of HpHypB-Ni(II) was solved at a resolution of 2.0 Å (Figure 3-12A). 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 centered on a metal ion. Each monomer in the structure of HpHypB adopts an α/β fold with a central 7-stranded parallel β-sheet sandwiched by α-helices, classical of the SIMIBI class of GTPases. It is similar to the structures of HypB from M. jannaschii and Archeoglobus fulgides, with a rmsd of 1.5 Å over 195 and 184 equivalent residues, respectively, considering monomers A.

The GTP analog GTPγS was included during crystallization, but surprisingly 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 the crystal growth step, because GTPγS can slowly hydrolyze over time, or contamination of the analog stock. The major difference between the two monomers is that the Mg(II) ion and P_i are 100 % occupied in monomer A while the occupancies are estimated to be approximately 60 % and 80 %, respectively, in monomer B. This difference seems to impact the stability of residues 82 to 85 in monomer B, which corresponds to a part of the switch I domain. Aside from this difference, the monomers are similar with a global rmsd value of 0.3 Å.

Concerning the presence of metal in HpHypB, a unique peak in the anomalous difference electron density map corresponding to one nickel ion at 100 % occupancy is present at the interface of the two monomers. Ni(II) is coordinated by Cys106 and Cys142 from monomers A and B, adopting a square-planar geometry (Figure 3-12B), while the two His107 are not involved in nickel coordination but rather in π-stacking interactions with Tyr146. This metal coordination is different to that observed in the MjHypB structure, in which two Zn(II) ions were ligated in tetrahedral geometries (called Zn_A and Zn_B). Zn_A is coordinated by Cys127 (corresponding to Cys142 in HpHypB) from monomer A, Cys95 (corresponding to Cys106 in HpHypB) from both monomers A and B and a water molecule. Zn_B is only coordinated by residues belonging to monomer B: Cys95, Cys127 and His96 (corresponds to His107 in HpHypB). A water molecule completes the coordination sphere of the metal. Superposition of the HpHypB and MjHypB metal-binding sites reveal that the nickel ion and Zn_A are very close and they are both positioned at the nickel site proposed by Chan et al. The main difference in the HpHypB metal-binding
site to that of Zn$_A$ in $Mj$HypB is the movement of Cys142$_B$ to complete the square planar coordination sphere of the Ni(II) ion (Figure 3-13A).

To facilitate a comparison, the $Mj$HypB-Zn(II) and $Hp$HypB-Ni(II) structures were superimposed (Figures 3-12C and 3-13B). The positions of GTP$_\gamma$S in $Mj$HypB and GDP + P$_i$ in $Hp$HypB are almost identical, although the P$_i$ binds to the protein differently than the gamma phosphate of the GTP analog. Nonetheless, the interactions of the protein with ribose and the $\alpha$- and $\beta$- phosphates are very similar. In the case of the guanine base, the interactions are different due to several non-conserved residues. For example, in regards to the [S/N]KXD base-recognition motif, Ser182 does not appear to interact with nucleotide in $Hp$HypB, while the corresponding residue in $Mj$HypB, Asn167, does. Secondary Structure Matching$^{48}$ revealed that there are no major differences between the G1 (P-loop/Walker A), G2 (Switch I) and G3 (Switch II/Walker B) domains in the two HypB structures. Together, these observations suggest that the “GDP + P$_i$” form may be considered as analogous to the “GTP” form found in $Mj$HypB rather than a “GDP” form.

Comparison of the Ni(II)-bound $Hp$HypB structure to that of the Zn(II)-bound $Mj$HypB revealed several slight but global re-arrangements (Figure 3-12C), particularly in two regions with larger rmsd values of about 2.4 Å. The first, residues 188-203, is positioned just after the G4 ([$S/N$]KXD) motif so it is connected to guanine binding. Conformational changes within this region may modulate the stability of the HypB homodimer, as it was previously demonstrated that M186 and F190 are involved in hydrophobic contacts at the dimeric interface and play a role in dimer stability.$^{26}$ The second region corresponds to residues 216 to 223, just after the G5 motif (the poorly conserved SAK sequence). These conformational changes impact guanine fixation, which is no longer directly bound to the protein as in $Mj$HypB, but via water molecules (Figure 3-13B), and also result in shifts of the secondary structural elements at the protein surface (Figure 3-12C).
Figure 3-12. The crystal structure of Ni(II)- and GDP + P_i-bound *Hp*HypB. (A) The structure of Ni(II)- and GDP + P_i-bound *Hp*HypB features a dimeric structure in which the two nucleotides are bound at the dimer interface along with a single nickel ion (in green). The two monomers A and B are colored cyan and purple, respectively. Nucleotides are depicted in orange and magnesium in pink. Thiolates involved in nickel coordination are depicted in yellow. (B) The nickel-binding site in *Hp*HypB consists of a tetrathiolate coordination sphere bridging the *Hp*HypB dimer with Cys106 and Cys142 contributed by both monomers. The nickel site in the crystal structure was shown in X-ray anomalous difference electron density map obtained at $\lambda_{\text{Se}} = 0.97969$ Å. The electron density peak contoured at 6 $\sigma$ was modeled as one nickel ion. (C) Superimposition of *Hp*HypB (in cyan and purple) and *Mj*HypB (in grey) crystal structures. The GDP + P_i of *Hp*HypB and GTP\gamma S of *Mj*HypB are colored in orange and black respectively. Residues 188 to 203 are depicted in magenta and residues 216 to 223 are depicted in yellow. These two groups of residues adopt different conformations in the Ni(II)-bound *Hp*HypB structure versus that of the Zn(II)-bound *Mj*HypB.
Figure 3-13. Overlay of *Hp*HypB and *Mj*HypB crystal structures. (A) Overlay of *Hp*HypB (monomer A in cyan, B in purple) and *Mj*HypB (in grey) metal-binding sites. (B) Overlay of *Hp*HypB (monomer A in cyan, B in purple) and *Mj*HypB (in grey) GTP-binding sites: main chains of residues 189 and 190 of *Hp*HypB interact with guanosine group using intermediate water molecules whereas corresponding residue main chains of *Mj*HypB interact directly.
**His107 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 His107. 9,29,32 In order 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 a LMCT band centered around 340 nm (Figure 3-14A 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, the addition of nucleotide to the mutant protein does not alter the energy of this band, in contrast to WT HpHypB. These data suggest that the change in the electronic absorption spectrum observed upon addition of nucleotide to WT HpHypB is due to loss of the imidazole ligand.

Mutation of His107 resulted in a dramatically weakened Ni(II) affinity of (2.89 ± 0.04) x 10^{-3} M, suggesting that it is a metal required ligand in the nucleotide-free protein. However, when the protein was loaded with nucleotide, the nickel affinity increased by several orders of magnitude to (5 ± 1) x 10^{-5} M when GDP-bound and < 10^{-5} M when GDPNP-bound (Figure 3-14A and Table 3-5). Further evidence of this increased metal-binding ability by H107A in the presence of nucleotide can be garnered from analytical gel filtration experiments. In contrast to wild-type HpHypB, the H107A mutant protein did not dimerize in the presence of 2 mM NiCl₂ alone, but upon the addition of 500 µM GDP a nearly complete shift to dimer was observed (Figure 3-15A). As a control, GDP did not induce dimer formation in the absence of metal (Figure 3-15A). A similar increase in dimer formation of the nickel-loaded protein was also observed in the presence of GDPNP (data not shown). Altogether, these data suggest that His107 is an essential nickel ligand only in the nucleotide-free state of the protein.
Figure 3-14. Metal binding to H107A and C142S HpHypB. (A) Ni(II) was titrated into a sample of 10 µM apo-H107A (black squares) and C142S HpHypB (grey 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 apparent $K_d$ of $(2.89 \pm 0.04) \times 10^{-3}$ M [$n = 1.3 \pm 0.1$] and $(1.7 \pm 0.5) \times 10^{-4}$ M [$n = 1.7 \pm 0.3$] for H107A and C142S HpHypB, respectively. In the presence of 500 µM GDP, the affinity of 5 µM H107A for nickel increases to an apparent $K_d$ of $(5 \pm 1) \times 10^{-5}$ M [$n = 1.0 \pm 0.2$] (black triangles), whereas the affinity of C142S in the presence of GDPNP is similar to that in the absence of nucleotide, with an apparent $K_d$ of $(3 \pm 1) \times 10^{-4}$ M [$n = 1.8 \pm 0.5$] (grey circles). Inset: The difference spectra of 20 µM C142S HpHypB (black line) and 10 µM H107A HpHypB (grey line) incubated with 1 mM and 800 µM NiCl$_2$, respectively, display LMCT bands at 340 nm. The extinction coefficient was calculated on the basis of the protein concentration. (B) The signal of the zinc-zincon complex at 620 nm was monitored to reveal that 140 µM ZnSO$_4$ is required to saturate 140 µM zincon (black diamonds). When 20 µM WT HpHypB was added, zinc binding to zincon was not observed until more than 20 µM ZnSO$_4$ was added, suggesting that there is one Zn(II) site on HpHypB that can outcompete zincon (black circles). Upon mutation of Cys142, 160 µM ZnSO$_4$ is still required to saturate the zincon, indicating that C142S (grey squares) HpHypB can still bind Zn(II) but with diminished affinity compared to WT.
Figure 3-15. Effect of metal and nucleotide on the quaternary structure of H107A and C142S HpHypB. (A) In the absence of metal, 50 µM H107A HpHypB eluted 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 (grey 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 grey line). The large peaks at 20-25 mL are due to free nucleotide. (B) In the absence of metal, 50 µM C142S HpHypB eluted at a volume corresponding to a monomer (solid grey line). The addition of 1 mM NiCl₂ (solid black line) induced a small amount of dimer formation, but significantly less than in WT, likely due to the weaker metal-binding ability of the C142S mutant. The chromatographic traces (monitored at 280 nm) are representative data sets from experiments with a Superdex 200 10/300 analytical column equilibrated with 25 mM HEPES (pH 7.6), 200 mM KCl, and 5 mM MgCl₂. The ticks at the top of the graph denote the elution volumes of the protein standards. From left to right, the identities of the standards (and their molecular masses) are thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B₁₂ (1.4 kDa), respectively.
**Cys142, not His107, couples metal binding to GTPase inhibition.** The observation that His107 coordination to nickel is modulated by nucleotide raised the possibility that this residue contributes to metal-mediated inhibition of GTP hydrolysis. To test this hypothesis, GTPase assays were conducted with the H107A mutant (Figure 3-16). The apo-H107A mutant featured two-fold diminished GTPase activity compared with apo-WT (Table 3-4), with the main change in behavior stemming from an increased $K_m$ in the mutant. This may be due to the similarly perturbed GTP-binding noted in nucleotide binding experiments (Table 3-7). However, the H107A mutant was still responsive to metal, with an overall decrease in enzymatic activity observed in the presence of Ni(II) and poor hydrolysis detected when Zn(II) was added (Table 3-4 and Figure 3-16). Overall, these results suggest that H107A does not serve as a metal sensor in *Hp*HypB.

Another metal-binding residue with a potential role in communicating the metal-bound state of *Hp*HypB to the GTPase activity is Cys142. This residue is a part of the Switch II motif, which typically undergoes a reorganization coupled to GTP hydrolysis and is thought to transduce

![Figure 3-16. GTPase activity of H107A HpHypB.](image)

**Figure 3-16. GTPase activity of H107A HpHypB.** Apo-H107A *HpHypB* (2 µM, circles) was incubated with increasing concentrations of GTP at 37 °C for 30 min in an anaerobic glovebox prior to detection of released inorganic phosphate using a colorimetric Malachite green assay. Fitting the data from several experiments to the Michaelis-Menten equation yields a $k_{cat}$ of $(8 \pm 1) \times 10^{-3}$ s$^{-1}$, a $K_m$ of $(1.3 \pm 0.2) \times 10^{-4}$ M, and a $k_{cat}/K_m$ of $62 \pm 11$ M$^{-1}$ s$^{-1}$. The presence of 500 µM NiCl$_2$ (squares) impaired the GTPase activity of the enzyme, resulting in a $k_{cat}$ of $(1.7 \pm 0.7) \times 10^{-3}$ s$^{-1}$, a $K_m$ of $(1.3 \pm 0.3) \times 10^{-4}$ M, and a $k_{cat}/K_m$ of $12 \pm 3$ M$^{-1}$ s$^{-1}$. Similarly, the addition of 500 µM ZnSO$_4$ (triangles) also impaired the enzyme, but to a greater degree. All datasets are the average of three independent replicates and error bars represent the standard deviation.
effector binding to altered GTP binding/hydrolysis. To test this possibility, Cys142 of 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 a LMCT shifted from that of WT protein, with a $\lambda_{\text{max}}$ at 340 nm (Figure 3-14A inset). In the nucleotide-free state, C142S HpHypB features diminished Ni(II) affinity, with an apparent $K_d$ of $(1.7 \pm 0.5) \times 10^{-4}$ M and a Hill coefficient of $1.7 \pm 0.3$ (Figure 3-14A and Table 3-5). The Hill coefficient is indicative of cooperative binding, which is likely due to the ability of this protein to dimerize, although to a significantly smaller extent than WT (Figure 3-15B). In contrast to H107A HpHypB, the addition of GDPNP does not drastically affect the affinity of C142S for Ni(II) (Table 3-5 and Figure 3-14A). The GDP bound C142S mutant, however, no longer displays any detectable LMCT bands, suggesting Ni(II) binding has been completely disrupted.

In order to determine if C142S still binds Zn(II) in the absence of nucleotide, a competition between C142S HpHypB and the metallochromic indicator zincon was established. In the absence of protein, metal loading of 140 µM zincon is complete upon addition of 140 µM Zn(II) (Figure 3-14B). Upon the inclusion of 20 µM WT HpHypB, Zn(II) binding to zincon is not detected until more than 20 µM metal has been added and 160 µM Zn(II) is required to saturate the indicator (Figure 3-14B). This result is consistent with the conclusion that WT HpHypB binds stoichiometric zinc with an affinity that is significantly greater than that of zincon. When the same competition was conducted with 20 µM C142S HpHypB, 160 µM Zn(II) was still required to achieve zincon saturation (Figure 3-14B), indicating that the C142S mutant of HpHypB maintains the ability to bind Zn(II), although the initial plateau region was less prominent than for wild-type HpHypB.

Given that the protein retains the ability to bind transition metals, GTPase assays were conducted on the C142S mutant to determine if removal of this ligand disconnects the metal-responsiveness in HpHypB. Initial attempts at conducting full Michaelis-Menten curves were not successful due to diminished affinity of C142S for GTP (Table 3-14), so only $k_{\text{cat}}$ measurements were performed. The $k_{\text{cat}}$ of the apo-C142S mutant is approximately four times lower than that of apo-WT (Table 3-4). In contrast to the significant inhibition observed with the wild-type protein, the addition of zinc only caused a 2-fold decrease in $k_{\text{cat}}$ in C142S HpHypB (Table 3-4). Similarly, the $k_{\text{cat}}$ of the mutant protein decreased approximately two-fold in the presence of nickel.
3.4 Discussion

The role of *HpHypB* in the biosynthesis of the two nickel-containing metalloenzymes in *H. pylori* requires functional GTP hydrolysis, and it is clear that the GTPase activity of this protein is intimately linked to selective interactions with several types of metals. The first is potassium, which accelerates GTP hydrolysis at concentrations in the range found within the *H. pylori* cytosol. In contrast, the GTPase activity is not affected by NaCl at concentrations as high as 100 mM, well past the physiologically relevant concentrations for Na⁺. Potassium-mediated improvement in GTP hydrolysis is an emerging trend, and was also observed with the GTPase involved in [FeFe]-hydrogenase maturation, HydF. For some of these GTPases, it is postulated that the potassium contributes to catalysis by neutralizing the developing negative charge on the phosphate during the reaction. It is also possible that potassium activates GTP hydrolysis by other mechanisms, such as structural stabilization and/or nucleotide binding, but neither of these properties of *HpHypB* are affected by potassium. Although potassium enhances the GTPase activity of *HpHypB*, the enzyme is still quite slow and other factors are likely to further stimulate GTP hydrolysis during metalloenzyme biosynthesis.

Although potassium does not dramatically alter any of the metal-binding or oligomeric properties of *HpHypB*, it does enhance the impact of transition metals on the GTPase activity, particularly that of nickel. In NaCl, Ni(II) decreases the *Kₘ* of the enzyme but simultaneously increases the *k₄₅*, resulting in a similar overall catalytic efficiency. However, when NaCl is replaced with KCl, Ni(II) has a clear inhibitory effect and hydrolysis is 34 times weaker than that observed with apo-*HpHypB*. In the case of zinc, the activity is significantly inhibited as previously observed, and although low levels of hydrolysis are detectable in the presence of potassium, they remain too weak to quantify. Zinc binding to *HpHypB* results in weaker nucleotide affinity, which may in part explain the inhibition of catalysis, but it is likely that other factors contribute to this effect.

In addition to nickel inhibiting GTP hydrolysis by *HpHypB*, it is clear that nucleotide impacts nickel binding to the protein. Nucleotide causes a change in the electronic absorption spectrum of the protein-nickel complex, suggesting a change in the coordination environment, as well as a decrease in metal stoichiometry from a 1:1 metal:protein complex to one metal ion per two protein monomers when loaded with nucleotide. The nickel stoichiometry observed in solution
is consistent with that observed in the crystal structure of nucleotide-loaded \( HpHypB \), which reveals a single nickel ion shared at a dimer interface. A similar decrease in metal stoichiometry was observed for \( E. coli \) YjiA (Chapter 4),\(^{35} \) a member of the same G3E family of GTPases to which \( HpHypB \) belongs,\(^{62} \) suggesting that this change in stoichiometry upon nucleotide binding may be a family-wide trait. Furthermore, in the case of \( HpHypB \), the nucleotide causes a distinct impact on the nickel affinity of the protein. Upon GDP binding, the Ni(II) affinity drops by more than an order of magnitude over that of the GTP-bound and nucleotide-free protein, leading to the model that GTP hydrolysis could trigger nickel release to subsequent steps of the biosynthetic pathways.

One major change in the nickel-binding site of \( HpHypB \) that is mediated by nucleotide loading is coordination by His107. Previous biochemical analysis of a \( HpHypB \) double mutant suggested that this conserved residue is involved in nickel binding,\(^{9} \) consistent with the observation that mutation of the corresponding His167 in \( EcHypB \) abrogates nickel binding to the GTPase domain of the protein.\(^{29} \) Furthermore, X-ray absorption spectroscopy data of nickel-loaded \( EcHypB \) was best fit to a model that included an imidazole ligand.\(^{32} \) These solutions studies were all conducted in the absence of nucleotide. In contrast, the crystal structure of nucleotide-loaded \( HpHypB \) reveals a tetrathiolate coordination site around the nickel ion, with Cys106 and Cys142 serving as metal ligands. Both His107 residues (one from each monomer) appear to be involved in an aromatic stacking interaction with Tyr146, a residue conserved in all bacterial HypB homologs as either a Tyr or Phe (Figure 3-17). In support of this nucleotide-mediated change in nickel coordination, analysis of the H107A \( HpHypB \) mutant revealed that this substitution has a much larger impact on nickel binding in the nucleotide-free protein. Loading the mutant protein with either GDP or GTP results in significantly stronger Ni(II)-binding activity, although the affinity for nickel is still at least 10 times weaker than that of WT \( HpHypB \) in the presence of nucleotide. The effect of His107 on the nickel affinity of the nucleotide-bound protein suggests that although His107 does not play a critical role in metal binding, it may still exert some influence, possibly via \( \pi \)-stacking with Tyr146. This latter residue is just downstream of the Switch II motif that includes the metal ligand Cys142, so the interaction between His107 and Tyr46 could influence nickel affinity and/or the nucleotide binding site, but additional work will be required to test this possibility.
In the case of zinc binding to HpHypB, there is no evidence for an analogous change in metal coordination. In contrast to the observation with nickel, a change in the Zn(II) stoichiometry of HpHypB in the presence of nucleotide is not observed, and only a minor effect on the affinity of the protein for zinc upon GDPNP loading was noted. Solution studies of HpHypB and EcHypB are consistent with His107 serving as a zinc ligand in the absence of nucleotide. Furthermore, the crystal structure of MjHypB loaded with Zn(II) and a GTP analog revealed that one histidine, His96, from one monomer acts as a Zn(II) ligand. In this structure, both the ligating and non-ligating His96 residues appear to be participating in aromatic stacking interactions with a nearby phenylalanine residue (Phe131, which corresponds with Tyr146 of HpHypB) as observed for

Figure 3-17. Amino acid alignment of HpHypB with homologs. The specific strains are as follows: Helicobacter pylori 26695, Helicobacter hepaticus ATCC 51449, Methanocaldococcus jannaschii DSM 2661, Escherichia coli K12 substr. MG1655, Bradyrhizobium japonicum USDA110, and Rhizobium leguminosarum. All sequences were retrieved from the NCBI database and aligned by using ClustalW.

In the case of zinc binding to HpHypB, there is no evidence for an analogous change in metal coordination. In contrast to the observation with nickel, a change in the Zn(II) stoichiometry of HpHypB in the presence of nucleotide is not observed, and only a minor effect on the affinity of the protein for zinc upon GDPNP loading was noted. Solution studies of HpHypB and EcHypB are consistent with His107 serving as a zinc ligand in the absence of nucleotide. Furthermore, the crystal structure of MjHypB loaded with Zn(II) and a GTP analog revealed that one histidine, His96, from one monomer acts as a Zn(II) ligand. In this structure, both the ligating and non-ligating His96 residues appear to be participating in aromatic stacking interactions with a nearby phenylalanine residue (Phe131, which corresponds with Tyr146 of HpHypB) as observed for
the corresponding histidines in the nickel-loaded *Hp*HypB structure. The fact that His107 coordinates zinc in the presence of nucleotide but not nickel suggests that it may play a role in distinguishing between the two metals.

Mutation of Cys142 to a non-ligating serine resulted in weakened metal binding to *Hp*HypB under all conditions investigated, suggesting this residue is always involved in metal coordination regardless of nucleotide-bound state. This result is consistent with the available structural data, as well as analysis of the corresponding C198A mutation in EcHypB, which disrupted nickel binding without abrogating it altogether. Replacing Cys142 with a non-ligating serine decouples the GTPase and metal-binding activities of *Hp*HypB. Zn(II) binding to the mutant protein yields a $k_{cat}$ only 2-fold lower than that of apo-C142S, instead of reducing the GTPase activity down to barely detectable levels as it does in the wild-type protein, and a similar impact of nickel binding on GTPase activity was also observed. This observation is consistent with the location of Cys142 on the Switch II motif, a critical region of GTPases responsible for transducing ligand-binding events to altered catalysis.

Cys142 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 more flexible in the GTP-bound state than in the GDP-bound state and often undergoes conformational rearrangement upon GTP hydrolysis. However, this general switch mechanism is modified depending on the specific role of the 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. The crystal structure of NifH revealed a conserved Cys residue in the Switch II motif that coordinates an Fe$_4$S$_4$ cluster. Analysis of the structure, which was loaded with ADP and AlF$_4^−$ in an effort to mimic the transition state, revealed that some of the Switch II motif connects binding of the AlF$_4^−$ to contacts with another subunit with the Fe$_4$S$_4$ cluster in between. This connection lead to the proposal that the nucleotide switch motif couples electron transfer and ATP hydrolysis to intersubunit stabilization. Another example involves the EF-Tu-related proteins, in which the Switch II motif mediates several specific interactions between the protein domains depending on nucleotide-bound state. In 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 also impact the interactions between HypB and other accessory proteins of the hydrogenase and urease biosynthetic pathways.

A comparison of the \(Hp\)HypB and \(Mj\)HypB 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 homologs (Figure 3-17), 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\(_i\) bound to \(Hp\)HypB corresponds to the GTP-bound state of \(Mj\)HypB. In many G-proteins, the enzyme is viewed as a molecular switch in which the GTP-bound state is thought of as an “ON state”, in which the protein is primed and ready for its physiological role.\(^{66}\) Some local differences between the \(Hp\)HypB and \(Mj\)HypB metal-bound structures were observed in the manner that the two proteins interact with the guanine base. In the \(Hp\)HypB structure, the regions involved in GTP binding are in a conformation allowing for better solvent accessibility, which may provide an explanation for the less dramatic inhibition of GTP hydrolysis than when zinc is bound.

Based on all available data, we propose that HypB plays a role in metal selectivity within the [NiFe]-hydrogenase maturation pathway (Figure 3-18). If we arbitrarily start the cycle with the GTP-bound species, the \(Hp\)HypB dimer can bind a single Ni(II) with nanomolar affinity. Although GTP hydrolysis is weak when Ni(II) is bound, another protein in the pathway could stimulate this activity. Recent studies with \(Ec\)HypB and SlyD demonstrated that SlyD accelerates GTPase activity in Ni(II)-bound HypB.\(^{52}\) It is also possible that interactions with other factors within the multiprotein nickel delivery complex, including the hydrogenase precursor protein itself, could result in acceleration of GTP hydrolysis. After hydrolysis, the GDP-bound state would more readily release the Ni(II) to the next step in the pathway, possibly to the hydrogenase enzyme in cooperation with HypA, and the GDP-bound protein would then recycle to the GTP-bound state. In the case of urease maturation, we propose that the Ni(II) is directed towards the nascent urease enzyme via HypA, depending on the cytosolic pH of the bacteria, as recently suggested.\(^{28}\) Such a pathway is supported by the observation that HypA interacts with UreE and this interaction is essential for urease maturation.\(^{67}\) Alternatively to Ni(II), if \(Hp\)HypB is loaded with Zn(II) it would no longer be able to hydrolyze the GTP to GDP. It is also possible that the \(Hp\)HypB conformation compelled by the distinct coordination environment of zinc, including ligation by His107, impacts protein-protein interactions during metallocenter
assembly. This combination of effects would prevent transfer of the non-cognate metal and preclude the protein from participating in any downstream steps, ensuring that only nickel is delivered to the nascent metallocenter. Thus, the interplay between nucleotide binding and metal binding in HpHypB may be a key aspect of the role of this protein within the hydrogenase biosynthetic pathway. Future research will reveal whether the properties of HpHypB described here are conserved in other homologs, and how the activities of the protein respond within the fully functional nickel insertion complex.

Figure 3-18. Proposed model of Ni(II) vs. Zn(II) discrimination by HpHypB. The GTP-loaded protein can bind a single Ni(II) ion with a nanomolar affinity. GTP hydrolysis may be stimulated by other components of the maturation pathway such as SlyD or the hydrogenase precursor protein. The resulting GDP-bound HpHypB possesses weaker Ni(II) affinity, accelerating Ni(II) donation. If Zn(II) binds to the GTP-bound protein instead the Zn(II)-bound 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. This prevents the transfer of Zn(II) to any downstream steps in the maturation of the enzyme metallocenter.
3.5 References


4 Metal-Binding Properties of \textit{Escherichia coli} YjiA, a Member of the Metal Homeostasis-Associated COG0523 Family of GTPases^4

4.1 Introduction

GTPases are molecular switches that contribute to a wide variety of critical cellular processes ranging from ribosomal protein synthesis to the cell cycle.\textsuperscript{1-3} The majority of GTPases are members of the phosphate-binding loop (P-loop) NTPase class that share a mononucleotide-binding fold composed of conserved nucleotide-binding motifs and a central, mostly parallel, \(\beta\)-sheet surrounded by \(\alpha\)-helices.\textsuperscript{4,5} All P-loop NTPases contain conserved structural elements including the P-loop (Walker A motif), the Walker B motif, and the switch I region, which are essential for binding and hydrolysis of GTP as well as for the ensuing conformational changes that trigger the downstream effects.\textsuperscript{2,4} The P-loop NTPases can be phylogenetically sorted into several families based on shared structural and sequence features.\textsuperscript{4} One such family is composed of the G3E NTPases, which possess a glutamate residue in place of the conserved aspartate in the Walker B motif, responsible for coordinating the catalytically essential magnesium ion.\textsuperscript{4} The G3E family features four main subfamilies, three of which (HypB, UreG, MeaB/ArgK) are based on prototypical proteins with established roles in the assembly of metalloenzyme active sites.\textsuperscript{6} The fourth subfamily, COG0523, is a more diverse and ubiquitous group of proteins with mostly unknown functions and a distribution across all three domains of life.\textsuperscript{4,6}

A recent phylogenetic study, which suggested that the COG0523 subfamily of P-loop GTPases can be separated into at least 15 subgroups, predicted that some members of this subfamily function in metal metabolism.\textsuperscript{6} For example, several subgroups were linked to zinc homeostasis.

\textsuperscript{4} Reprinted with permission from Sydor, A. M., Jost, M., Ryan, K. S., Turo, K. E., Douglas, C. D., Drennan, C. L., and Zamble, D. B. (2013) Metal Binding Properties of \textit{Escherichia coli} YjiA, a Member of the Metal Homeostasis-Associated COG0523 Family of GTPases, \textit{Biochemistry} 52, 1788-1801. Copyright 2013 American Chemical Society. Author contributions: A.M.S. performed the experiments except for mass spectrometry and crystallography, analyzed data and wrote the paper. M.J., K.S.R., K.E.T. conducted the crystallography and analyzed the resulting data, C.D.D. performed the mass spectrometry experiments, M.J., C.L.D, and D.B.Z. wrote the paper. The main text has not been changed, but reformatted for consistency within this thesis. All tables and figures including data presented as supplementary information have been added to the main text and renumbered.
because of promoters containing the DNA recognition sequence of Zur, a zinc-responsive transcription factor, consistent with the zinc-dependent regulation observed for COG0523 members from several organisms. 6-9 In addition, there are a few reports about individual COG0523 proteins that support specific roles in transition metal pathways. The first member of this subfamily identified was *Pseudomonas denitrificans* CobW, a protein essential for cobalamin biosynthesis. 10 The function of CobW is ambiguous, but it has been suggested that it is responsible for delivery of cobalt to the cobaltochelatase during assembly of the metallocofactor. 11-13 Another COG0523 member is the nitrile hydratase activator protein Nha3, which is involved in the biosynthesis of the iron-dependent nitrile hydratase in *Rhodococcus* sp. N-771 and is proposed to be responsible for trafficking the proper metal to the enzyme precursor. 14 A third described COG0523 factor is *Bacillus subtilis* YciC. Originally reported as a low-affinity zinc transporter, 7 YciC has since been proposed to serve as a metallochaperone for delivery of the metal cofactor into YciA, a back-up enzyme involved in folate biosynthesis. 15 Both YciC in *B. subtilis* 7 and the more recently reported YeiR from *E. coli* 16 help their host bacteria survive in zinc-limited growth conditions. Furthermore, in vitro analysis of YeiR revealed a link between metal binding and the protein’s oligomerization and GTPase activities. 16

Despite the large size of this subfamily and its connection with metal homeostasis, very little is known about the biochemical properties of the COG0523 constituents. The only high-resolution structural information on this subfamily is provided by the crystal structure of apo-YjiA from *E. coli*, 17 making this protein an attractive target for structure/activity analysis. To garner further information into the metal-binding activities of the COG0523 proteins and to better understand the possible connection with metal homeostasis, we carried out a bioinorganic characterization of YjiA. Metal-binding studies demonstrate that YjiA can bind stoichiometric cobalt, two equivalents of nickel, or four zinc ions in solution, and loading the protein with metal inhibits the GTPase activity. Using X-ray crystallography and spectroscopy, we identified a unique metal-binding site that, unlike those found in the UreG or HypB subfamilies, 18, 19 is located in a solvent-accessible pocket formed by a cysteine and two glutamate residues. This metal-binding site is adjacent to the nucleotide-binding site and is likely responsible for the observed effects of metal on GTP hydrolysis by YjiA. This report furthers our understanding of this large class of proteins, supporting a role for metal-dependent regulation, and will be useful in dissecting the function of the uncharacterized COG0523 proteins.
4.2 Materials and Methods

**Materials.** Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs. Primers (Table 4-1) were purchased from Sigma Genosys. All chromatography media were from GE Healthcare. Kanamycin, tris(2-carboxyethyl)phosphine (TCEP), phenylmethylsulfonyl fluoride (PMSF), and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from BioShop (Toronto, ON). Nickel chloride, zinc sulfate, and cobalt sulfate salts (as a minimum, 99.9 % pure) were purchased from Aldrich. All other metal stocks were atomic absorption standard solutions. All other reagents were analytical or molecular biology grade from Sigma-Aldrich. The buffers for all metal assays were treated with Chelex-100 (Bio-Rad) to minimize trace metal contamination. All samples were prepared with Milli-Q water, 18.2 MΩ-cm resistance (Millipore).

**WT and Mutant YjiA Expression Vector Construction.** The *yjiA* gene was amplified from genomic *E. coli* (DH5α) DNA using primers (Table 4-1) designed with restriction sites for *NdeI* (*YjiA forward*) and *XhoI* (*YjiA reverse*). The digested PCR product was ligated with T4 DNA

### Table 4-1: PCR primers used for cloning and mutagenesis.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>YjiA forward</td>
<td>5’GGAGACCATATGAAAAACTGCATTGCTACGTCATC’5'</td>
</tr>
<tr>
<td>YjiA reverse</td>
<td>5’GCCCATCTCGAGTTCTTCCTCAACC’5’</td>
</tr>
<tr>
<td>YjiA C66A,C67A forward</td>
<td>5’CTGACCAAACGGGTGCATCGTCGTCGCTCAACC’5’</td>
</tr>
<tr>
<td>YjiA C66A,C67A reverse</td>
<td>5’GCTCGTTGGAGCGCGATGTCGAGCCCTTGATCGAC’5’</td>
</tr>
<tr>
<td>YjiA E42A forward</td>
<td>5’GATTGAAAACGAATTCGCCCAGTCTCTGTTGATGATC’5’</td>
</tr>
<tr>
<td>YjiA E42A reverse</td>
<td>5’GATCATCAACAGAGACTggGCCGAATTCGTTTCACCTTC’5’</td>
</tr>
<tr>
<td>YjiA E37A forward</td>
<td>5’CAAGATTGCGCCGTGATTGcAAACGAATTCGCGAAG’5’</td>
</tr>
<tr>
<td>YjiA E37A reverse</td>
<td>5’CTTCGCGCAATTCGTTTcGAATCAGCGCAATTCGTTT’5’</td>
</tr>
</tbody>
</table>

*Restriction enzyme sites are shown in bold. Mutations are shown in lowercase. The E42A,C66A,C67A and E37A,C66A,C67A mutants were constructed using the C66A,C67A YjiA-pEt24b vector as a template and the E42A and E37A primers, respectively.*
ligase into the pET24b vector (Novagen) digested with NdeI and XhoI and dephosphorylated with calf intestinal phosphatase (New England Biolabs). The mutations were introduced into the YjiA-pET24b vector using QuikChange PCR mutagenesis (Stratagene) with Pfu Turbo DNA polymerase and the primers in Table 4-1. The template was subsequently digested with DpnI. The E42A,C66A,C67A and E37A,C66A,C67A triple mutants were constructed by using the C66A,C67A YjiA-pET24b vector as a template and the E42A and E37A primers, respectively.

For production of large amounts of the wild type (WT) and mutant plasmids, the plasmids were transformed into XL-2 Blue E. coli (Stratagene) and isolated using the Fermentas GeneJET plasmid miniprep kit. All plasmids were sequenced (ACGT, Toronto, ON) in the forward and reverse directions.

**Protein Expression and Purification.** For expression of WT and mutant YjiA, the plasmids were transformed into BL21 Star (DE3) E. coli cells (Novagen). Overnight cultures were grown and 25 mL was used to inoculate 1.5 L of LB medium supplemented with 50 µg/mL kanamycin. The cells were grown aerobically at 37 °C until the OD_{600} reached 0.6, at which point they were induced with 1 mM IPTG. After incubation at 37 °C for 5 h, the cells were harvested by centrifugation and resuspended in 20 mM Tris, pH 7.5, and 100 mM NaCl supplemented with 4 mM TCEP and 200 µM PMSF. For each preparation of purified protein, a total of 9 L of cell culture was used. All subsequent steps were performed at 4 °C or on ice. The resuspended cells were sonicated and centrifuged at 25,000 × g for 40 min. The supernatant was passed through a 0.45 µm syringe filter and then loaded onto a DEAE Sepharose anion-exchange column (GE Healthcare) equilibrated with buffer A (20 mM Tris, pH 7.5, and 1 mM TCEP). Fractions from a NaCl gradient were screened by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5 % gels. WT and mutant YjiA eluted at approximately 250 mM NaCl. Fractions containing the protein of interest were pooled and dialyzed against buffer A. The sample was then loaded onto a HiTrapQ anion-exchange column (GE Healthcare) equilibrated with buffer A. Once again, fractions from a NaCl gradient were screened by SDS-PAGE and fractions containing the protein of interest (eluted at approximately 150 mM NaCl) were pooled. Following concentration of the pooled fractions to 1 mL using Amicon Ultra 3K MWCO centrifuge concentrators (Millipore), the protein was loaded onto a Superdex 200 gel filtration column (GE Healthcare) equilibrated with 25 mM HEPES, pH 7.6, 200 mM NaCl and 1 mM TCEP. Fractions containing the protein of interest were pooled, concentrated such that the final
concentration was in the range of 250 to 500 µM, and stored at -80 °C. The protein concentrations were calculated by using an extinction coefficient of 26,930 M⁻¹ cm⁻¹ at 280 nm for both YjiA and the YjiA mutants in 25 mM ethylenediaminetetraacetic acid (EDTA) and 4 M guanidinium hydrochloride (GuHCl). Unless otherwise noted, electronic absorption measurements were conducted on an Agilent 8453 spectrophotometer with a 1 cm pathlength cuvette. Each protein was analyzed by electrospray-ionization mass spectrometry (ESI-MS; Department of Chemistry, University of Toronto) and the observed molecular weights of the proteins are listed in Table 4-2. All proteins were > 90 % pure as estimated by Coomassie-stained SDS-PAGE quantified by using the public domain ImageJ program (U.S. National Institutes of Health [http://rsb.info.nih.gov/ij/]).

**Preparation of Proteins.** Reduced apo-protein was produced by incubating the protein with 10 mM EDTA and 20 mM TCEP in a Coy anaerobic glovebox at 4 °C for 48 h. The TCEP and EDTA were removed by exhaustive dialysis into protein buffer (25 mM HEPES, 100 mM NaCl, pH 7.6). The absence of any bound metal to the protein was confirmed by a 4-(2-pyridylazo)-resorcinol (PAR) assay, in which the protein was denatured with 4 M GuHCl and 50 µM PAR was added to the sample. The absorbance at 500 nm, due to the formation of the (PAR)₂Me(II) complex, was monitored and compared to a standard curve prepared with 50 µM PAR in 4 M GuHCl and known metal concentrations. The free thiol content of the proteins was quantified via reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in the presence of 6 M GuHCl 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 > 95 % reduced after treatment with TCEP.

**Table 4-2:** Calculated and observed molecular masses (MM) for WT YjiA and mutants, as determined by ESI-MS.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Calculated MM (Da)</th>
<th>Observed MM (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT YjiA</td>
<td>35659.6</td>
<td>35660.0</td>
</tr>
<tr>
<td>YjiA C66A,C67A</td>
<td>35595.5</td>
<td>35596.0</td>
</tr>
<tr>
<td>YjiA E37A,C66A,C67A</td>
<td>35537.5</td>
<td>35538.0</td>
</tr>
<tr>
<td>YjiA E42A,C66A,C67A</td>
<td>35537.5</td>
<td>35538.0</td>
</tr>
</tbody>
</table>
**Metal Binding and Stoichiometry.** For the difference spectra, individual samples containing 20 µM apo-YjiA in protein buffer and either 20 µM NiCl₂ or 20 µM CoSO₄ were prepared in the glovebox 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. The difference spectrum for each metal was generated by subtracting the apo-YjiA spectrum from that of the metal-loaded protein.

Metal stoichiometry experiments were conducted by incubating 120 µM WT or mutant apo-YjiA with either 480 µM NiCl₂, 360 µM CoSO₄, or 600 µM ZnSO₄ overnight at 4 °C in the glovebox. Samples containing mixtures of metal were similarly prepared with 120 µM WT apo-YjiA and either 360 µM NiCl₂ and 360 µM CoSO₄; 600 µM ZnSO₄ and 480 µM NiCl₂; or 600 µM ZnSO₄ and 360 µM CoSO₄. Samples with GDP contained 1.5 mM GDP and 5 mM MgCl₂. Excess metal was removed by passing the protein through a PD10 gel filtration column (GE Healthcare) equilibrated with protein buffer in the glovebox. The protein concentration was subsequently measured in GuHCl and EDTA, as described above, and the metal content was determined via a PAR assay. In the case of the YjiA samples containing a mixture of metals, a high-pressure liquid chromatography (HPLC)-based method for the detection and identification of metal ions in solution was utilized.²² For HPLC analysis, at least 50 µg of protein was dried by centrifugation under vacuum, reconstituted with metal-free concentrated HCl (SeaStar Chemicals), and incubated overnight at 95 °C for protein hydrolysis. The sample was once again dried to remove HCl and reconstituted in 80 µL of MilliQ water. This sample was injected onto a Dionex IonPak CS5A column, equilibrated with 7 mM pyridine-2,6-dicarboxylic acid, 66 mM KOH, 5.6 mM K₂SO₄, and 74 mM HCOOH, attached to a metal-free Dionex BioLC HPLC system. The metals were detected at 500 nm following post-column mixing with PAR.

**ESI-MS Measurements.** Before mass spectrometry experiments, 120 µM WT apo-YjiA was incubated with 600 µM ZnSO₄ overnight at 4 °C in the glovebox, then buffer exchanged into 10 mM ammonium acetate, pH = 7.5, using two consecutive PD10 columns. Mass spectra were acquired on an AB/Sciex QStarXL mass spectrometer equipped with an ion spray source in the positive mode and a hot source-induced desolvation interface. Ions were scanned in the 1000 – 2000 m/z range with accumulation times of one second per spectrum, with no interscan time delay. The instrument parameters were as follows: ion source temperature 200 °C; ion source gas 50.0 psi; curtain gas 50.0 psi; ion spray voltage 5000 V; declustering potential 60.0 V; focusing
potential 60.0 V; MCP (detector) 2200 V. The spectra were deconvoluted using the Bayesian protein reconstruction program included with the Analyst QS software (AB/Sciex) over a mass range of 30000-40000 Da, with a step mass of 1 Da.

**YjiA Metal Affinities.** To determine the affinity of YjiA for Ni(II), 250 nM YjiA in protein buffer was titrated with a nickel atomic absorption standard solution and the absorbance at 340 nm was monitored using a 10 cm pathlength cuvette and a GBC Cintra 404 spectrophotometer. The apparent $K_d$ was calculated by determining the fractional saturation, $r$, and free nickel concentration, $[\text{Ni(II)}]_{\text{free}}$, by using equations 1 and 2, respectively:

$$ r = \frac{[\text{YjiA-Ni(II)}]}{2 \times [\text{YjiA}]_{\text{total}}} = \frac{A_{340} - A_{\text{min}}}{A_{\text{max}} - A_{\text{min}}} \quad (1) $$

$$ [\text{Ni(II)}]_{\text{free}} = [\text{Ni(II)}]_{\text{total}} - (r \times 2 \times [\text{YjiA}]_{\text{total}}) \quad (2) $$

where $[\text{YjiA-Ni(II)}]$ is the concentration of protein bound to Ni(II), $[\text{YjiA}]_{\text{total}}$ is the total protein concentration, $A_{340}$ is the absorbance at 340 nm for a given Ni(II) concentration, $A_{\text{min}}$ is the absorbance at 340 nm for apo-YjiA, $A_{\text{max}}$ is the absorbance at 340 nm upon saturation, and $[\text{Ni(II)}]_{\text{total}}$ is the total Ni(II) concentration added to the sample. The resulting values were plotted as $r$ vs. $[\text{Ni(II)}]_{\text{free}}$, and the data fit to the Hill Equation (3):

$$ r = \frac{[\text{Ni(II)}]_{\text{free}}^n}{K_d^n + [\text{Ni(II)}]_{\text{free}}^n} \quad (3) $$

where $n$ is the Hill coefficient.

To determine the affinity of YjiA for Co(II), 5 µM apo-YjiA was titrated with CoSO$_4$ and the absorbance at 350 nm was monitored. The Co(II) $K_d$ was calculated in the same manner as for Ni(II), except that the equations 1 and 2 were adjusted to solve for a single metal site instead of two.

**YjiA Zincon Zn(II) Competitions.** In order to estimate the Zn(II) $K_d$ of YjiA, the competitor zincon was selected due to its ability to form a 1:1 complex with Zn(II) with a reported $K_d$ of
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 nM zincon in protein buffer with increasing amounts of ZnSO₄. The absorbance at 620 nm was monitored using a 10 cm pathlength cuvette and a GBC Cintra 404 spectrophotometer. The $K_d$ was calculated by using the same method as the Ni(II) and Co(II)-YjiA titrations, and with $n = 1$. Competition experiments were prepared by incubating 10 µM apo-WT or E37A,C66A,C67A YjiA with 140 µM zincon and various amounts of Zn(II). The samples were incubated overnight at 4 °C in the glovebox. The absorbance of zincon at 620 nm was monitored using a 2 mm pathlength cuvette.

**Analytical Gel Filtration Chromatography.** Samples containing 60 µM YjiA were incubated with the desired metal, GDP, or GTP at the indicated concentrations (Table 4-5) overnight at 4 °C in the glovebox. For samples containing GTP, the protein was pre-incubated with GTP for at least 2 hours at 4 °C in the glovebox prior to injection onto the gel filtration column. All samples contained 25 mM HEPES, pH 7.6, 100 mM NaCl, and 5 mM MgCl₂. Apo-protein and metal-containing samples were loaded onto a Superdex 200 10/300 analytical gel filtration column (GE Healthcare), pre-equilibrated with chelaxed and filtered 25 mM HEPES, pH 7.6, 200 mM NaCl, and 5 mM MgCl₂. The column was calibrated with thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.4 kDa) from BioRad. The inclusion of metal in the standards did not affect their elution profiles (data not shown). Molecular masses were determined by plotting the log molecular masses of the standards versus the partition coefficient ($K_{av}$), where $K_{av} = (V_e - V_o)/(V_t - V_o)$; $V_e$ represents the elution volume, $V_o$ is the void volume, and $V_t$ is the total column volume.

**Circular Dichroism (CD) Spectroscopy.** WT and mutant YjiA samples were prepared for CD spectroscopy by diluting the protein in MilliQ water to a final concentration of approximately 10-20 µM in the glovebox. For metal titrations, ZnSO₄, CoSO₄, or NiCl₂ was added to the diluted samples and allowed to equilibrate overnight at 4 °C in the glovebox. All samples were analyzed on an Olis RSM 1000 spectropolarimeter with a capped 1 mm pathlength cuvette in order 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 sec and 2400 grating lines per nm. The final spectra obtained are averages of three scans. The observed ellipticity was converted into mean residue ellipticity ($[\theta]_{mre}$; deg cm² dmol⁻¹) using the following formula:²⁵
where \( MW \) is the molecular weight of the protein in Da, \( N \) is the number of amino acids, \( \theta \) is the observed ellipticity in degrees, \([\text{protein}]\) is the concentration of protein in g/mL and \( l \) is the pathlength. The secondary structure content was quantitated in the 200-240 nm range using the K2D3 program.\footnote{26} The CD spectra of the mutants were similar to that of the WT protein, demonstrating that these mutations do not affect the secondary structure of the protein (data not shown).

**GTPase Assay.** GTPase activity was determined by the Malachite Green assay for free phosphate adapted from Lanzetta et al.\footnote{27} A series of 400 µL samples containing 0.5-2 µM WT or E37A,C66A,C67A YjiA in protein buffer supplemented with 5 mM Mg(II) and concentrations of GTP between 5 and 950 µM were incubated at 37 °C in the glovebox for 2.5-5 h. Control samples without protein were prepared alongside the protein samples and received the same treatment. After incubation, 100 µL of the freshly prepared 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 then mixed by vortexing and incubated at RT for 3 min, after which sodium citrate, pH was added to a final concentration of 3.5%. The samples were vortexed again and incubated at RT for 30 min prior to plating on a 96-well plate and measuring the absorbance at 630 nm with a Tecan Safire2 microplate reader. The amount of phosphate released was determined via a standard curve based on a phosphate standard (Molecular Probes). The data were analyzed by fitting to the Michaelis-Menten equation using OriginPro 7.5. Samples containing metal were pre-incubated with the metal overnight prior to the assay (prepared as a stock of 10-40 µM protein with 100 µM Zn(II), 100 µM NiCl\(_2\), or 250 µM CoSO\(_4\) for WT and 225 µM Zn(II), 400 µM NiCl\(_2\), or 800 µM CoSO\(_4\) for the E37A,C66A,C67A triple mutant). These stocks were then diluted to the final protein concentration of 0.5-2 µM for the assay in a buffer that contained 100 µM Zn(II), 100 µM NiCl\(_2\), or 250 µM CoSO\(_4\) for WT or 225 µM Zn(II), 400 µM NiCl\(_2\), or 800 µM CoSO\(_4\) for the E37A,C66A,C67A triple mutant.

**Crystallization and Zn(II) soaks.** WT YjiA and E37A,C66A,C67A YjiA were crystallized at 25 °C by the hanging drop vapor diffusion technique. For WT YjiA, 1.5 µL of a protein solution
(13.2 mg/mL in 100 mM NaCl, 25 mM HEPES, pH 7.6) were mixed with 0.3 µL 100 mM CaCl₂ and 1.5 µL of a precipitant solution (1.55 M (NH₄)₂SO₄, 0.1 M HEPES, pH 6.9) on a glass cover slip. The cover slip was sealed with grease over a reservoir containing 500 µL of the precipitant solution. Crystals appeared overnight and grew to maximum dimensions of about 300 × 50 × 50 µm after 3 days. Crystals of E37A,C66A,C67A YjiA were grown in the same fashion with the following modifications: the precipitant solution was composed of 1.3 – 1.35 M (NH₄)₂SO₄, 0.1 M HEPES, pH 7.5 – 7.7, the protein concentration was 12.4 mg/mL, and the protein solution contained 1 mM TCEP in addition to the other components.

Since co-crystallization of WT YjiA with Zn(II) was unsuccessful, Zn(II)-soaked WT YjiA crystals were generated by incubating apo-WT crystals in the precipitant solution supplemented with 3 mM ZnSO₄ and 1 mM TCEP at 25 °C for 16 h. The crystals were then directly transferred to a cryogenic solution (1.5 M (NH₄)₂SO₄, 0.1 M HEPES, pH 6.9, 3 mM ZnSO₄, 1 mM TCEP, 20% (v/v) glycerol), incubated for 5 s, and flash-frozen in liquid nitrogen. Zn(II)-soaked E37A,C66A,C67A YjiA crystals were generated in a similar fashion with the following modifications: the soaking solution contained an additional 20 mM CaCl₂ to better stabilize the crystals, the cryogenic solution consisted of 1.35 M (NH₄)₂SO₄, 0.1 M HEPES, pH 7.7, 20 mM CaCl₂, 3 mM ZnSO₄, 1 mM TCEP, 20% (v/v) glycerol, and the crystals were transferred into the cryogenic solution in 3 steps of increasing glycerol concentration and incubated in that solution for 20 s.

**Data collection.** All Zn(II)-soaked YjiA crystals belong to space group \( P2_1 \) (\( a \approx 56 \text{ Å}, b \approx 69 \text{ Å}, c \approx 78 \text{ Å}, \beta \approx 104° \)) and contain two protomers in the asymmetric unit. All diffraction data were collected at a temperature of 100 K. Diffraction data for Zn(II)-soaked WT YjiA were collected at the Stanford Synchrotron Radiation Laboratory beamline 9-2 in Portola Valley, CA, using a MARmosaic 325 CCD detector. An initial Zn(II)-soaked WT YjiA crystal was used for an X-ray fluorescence scan to verify the presence of bound Zn(II). Based on the fluorescence scan, a peak wavelength of 1.2827 Å (9665.5 eV) was determined using \( \text{CHOOCH} \). A wavelength of 1.1808 Å (10500.0 eV) was chosen for the remote data set. Remote and peak data sets were collected on the same crystal in wedges of 30° in 1° oscillation steps. For each wedge, the corresponding images related by a 180° crystal rotation were collected immediately after completion of the wedge. An inflection data set was also collected on the same crystal, but not
used for this study. Bijvoet mates were treated as separate reflections for both the remote and the peak data set.

Diffraction data for Zn(II)-soaked E37A,C66A,C67A YjiA were collected at the Advanced Photon Source beamline 24ID-C in Argonne, IL, using a Pilatus 6M pixel detector. The crystal was annealed for 5 s prior to data collection. Then, a single data set was collected at a wavelength of 0.9795 Å (12658.0 eV) in 1° oscillation steps. For structure solution, Bijvoet mates were treated as symmetry-related reflections. For calculation of Zn anomalous difference Fourier maps of Zn(II)-soaked E37A,C66A,C67A YjiA, Bijvoet mates were treated as separate reflections.

All data were integrated using HKL2000 and scaled using Scalepack. The same reflections were marked for the free set of reflections for Zn(II)-soaked WT YjiA and for Zn(II)-soaked E37A,C66A,C67A YjiA. In addition, 5% of the reflections in the resolution ranges of 75 – 30 Å and 2.57 – 2.05 Å were included in the free set for the data of the triple mutant. All data collection statistics are summarized in Table 4-3.

**Structure building and refinement.** The crystal structure of Zn(II)-soaked WT YjiA was determined at 2.57 Å resolution by molecular replacement with the crystal structure of native WT YjiA without water molecules (PDB ID 1NIJ17) in PHASER, which yielded a single solution. The output model was subjected to 20 cycles of simulated annealing in PHENIX to reduce model bias. The model was adjusted for any changes that had occurred through a cycle of model building in COOT followed by refinement in PHENIX. Initial difference electron density maps revealed the presence of 5 Zn(II) ions in the asymmetric unit. Zn(II) ions and water molecules were inserted in subsequent rounds of refinement.

The intermediate model of Zn(II)-soaked WT YjiA without water molecules or Zn(II) was used to determine the crystal structure of Zn(II)-soaked E37A,C66A,C67A YjiA to 2.05 Å resolution. The structure was solved by rigid body refinement in PHENIX followed by automated rebuilding of the structure using phenix.autobuild to reduce model bias. Initial difference electron density maps revealed the presence of all 3 mutations as well as the presence of 3 Zn(II) ions in the asymmetric unit. Subsequently, Zn(II) ions and water molecules were inserted and the model was adjusted to account for the mutations as well as other changes through iterative cycles of model building in COOT and refinement in PHENIX.
Due to the superior quality of the Zn(II)-soaked E37A,C66A,C67A YjiA data set (Table 4-3), the model derived from this data set was then used to finalize the structure of Zn(II)-soaked WT YjiA. Following rigid body refinement of the mutant structure into the WT YjiA data in PHENIX, additional Zn(II) ions were inserted and the structure was completed through iterative cycles of model building in COOT and refinement in PHENIX.

For both structures, the two protomers in the asymmetric unit were initially restrained by strict non-crystallographic symmetry (NCS) parameters. In advanced stages of refinement, NCS restraints were loosened for all residues and removed for residues involved in crystal contacts or Zn(II)-binding. Side chains with limited electron density were truncated at the last atom with visible electron density. Zn(II)-coordination was loosely restrained to average bond lengths observed in previously determined structures of proteins with Zn(II) ions bound. The final stages of refinement for both structures involved TLS parameterization using two TLS groups per protomer, as determined by the TLSMD server. The locations of Zn(II) ions were verified by Zn anomalous difference Fourier maps calculated from the peak data set (WT YjiA) or the remote data set (E37A,C66A,C67A YjiA) using the program FFT, which is part of the CCP4 program suite.

Crystallographic refinement of both structures yielded models that possess excellent stereochemistry and small root-mean-square deviations from ideal values for bond lengths and bond angles. Due to the presence of pseudo-translational symmetry in the data, the crystallographic R-factors are relatively high, but within an acceptable range. Refinement statistics are summarized in Table 4-3. The final model of WT YjiA contains all 318 residues of YjiA in protomer A and residues 2 – 318 in protomer B. The final model of E37A,C66A,C67A YjiA contains all 318 residues in both protomers. The models were validated using simulated annealing composite omit maps calculated in CNS. The geometries of the final models were analyzed with Molprobity. Figures were generated using Pymol.
Table 4-3. Crystallographic data collection and refinement statistics.

<table>
<thead>
<tr>
<th></th>
<th>Zn(II)-soaked WT YjiA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Zn(II)-soaked E37A,C66A,C67A YjiA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data collection</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Space group</td>
<td>$P2_1$</td>
<td>$P2_1$</td>
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<tr>
<td>Unit cell parameters</td>
<td></td>
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<tr>
<td>$a$, $b$, $c$ (Å)</td>
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<td>56.07, 68.44, 77.70</td>
</tr>
<tr>
<td>$\alpha$, $\beta$, $\gamma$ (°)</td>
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<td>90, 104.23, 90</td>
</tr>
<tr>
<td>Resolution (Å)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.0 – 2.57 (2.61 – 2.57)</td>
<td>75.3 – 2.05 (2.09 – 2.05)</td>
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<td>Unique reflections&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35529 (1535)</td>
<td>35715 (1784)</td>
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<tr>
<td>$R_{sym}$ (%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.9 (41.2)</td>
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<td>$&lt;I&gt;/\sigma(I)$&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.3 (3.3)</td>
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<td>Completeness (%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>97.0 (84.6)</td>
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<td>Multiplicity&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Wavelength (Å)</td>
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<td><strong>Refinement</strong></td>
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<td>Resolution range (Å)</td>
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<td>Rotamer outliers (%)</td>
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<sup>a</sup> For this data set, Bijvoet pairs were treated as separate reflections

<sup>b</sup> Values in parentheses indicate highest resolution bin

<sup>c</sup> r.m.s.d. is root mean squared deviation
4.3 Results

**YjiA binds Zn(II), Ni(II), and Co(II) with micromolar affinities.** The presence of a CXCC motif in YjiA, conserved in all COG0523 proteins, led to the proposal that the protein can bind metals, but experimental evidence was lacking. Due to the homology of YjiA to G3E NTPases known to be involved in Zn(II) (YeiR and YciC), Ni(II) (UreG and HypB), or Co(II) (CobW) homeostasis, these three metals were the focus of this investigation. To determine whether YjiA can bind these metals and, if so, how much, 120 µM apo-YjiA was incubated with either 480 µM NiCl$_2$, 360 µM CoSO$_4$, or 600 µM ZnSO$_4$ and excess metal was removed by gel filtration chromatography. Subsequent metal analysis via a PAR assay revealed roughly stoichiometric cobalt, 2 Ni(II), or 4 Zn(II) bound per monomer (Table 4-4).

After incubation of YjiA with 1 equivalent of nickel or cobalt, broad charge-transfer bands appear in the electronic absorption spectrum region of 250-400 nm (Figure 4-1A). The difference spectra, obtained by subtracting the signal of apo-YjiA from that of the protein loaded with 1 equivalent of Ni(II) or Co(II) (Figure 4-1A), reveal a peak maximum at 280 nm and a broad shoulder around 340 or 350 nm for nickel and cobalt, respectively. The 280 nm and shoulder absorptions for both metals can be attributed to Cys$\rightarrow$Ni(II)/Co(II) ligand-to-metal charge transfer (LMCT), indicating the presence of at least one thiolate in the coordination sphere of both metals.

<table>
<thead>
<tr>
<th>YjiA Variant</th>
<th>Ni(II) Bound</th>
<th>Co(II) Bound</th>
<th>Zn(II) Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>2.2 ± 0.2</td>
<td>1.2 ± 0.2</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>WT + GDP</td>
<td>1.3 ± 0.1</td>
<td>0.6 ± 0.2</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>E37A,C66A,C67A</td>
<td>0.5 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>3.9 ± 0.5</td>
</tr>
<tr>
<td>E42A,C66A,C67A</td>
<td>1.2 ± 0.2</td>
<td>0.2 ± 0.1</td>
<td>4.2 ± 0.3</td>
</tr>
</tbody>
</table>

*Apologies for 120 µM (120 µM) was incubated with either 480 µM NiCl$_2$, 360 µM CoSO$_4$, or 600 µM ZnSO$_4$ overnight at 4 °C in an anaerobic glove box. Excess metal was removed by passing the proteins over a PD10 gel filtration column and the bound metal was detected via a PAR assay. In samples containing GDP, 1.5 mM GDP and 5 mM MgCl$_2$ were included. The data listed are average values and standard deviations of the number of metal ions bound per protein monomer from at least three independent experiments.
The LMCTs for Ni(II) and Co(II) can be used to monitor metal binding by YjiA. A titration of 5 μM apo-YjiA with CoSO₄ yields an apparent $K_d = (2.0 \pm 0.1) \times 10^{-5}$ M and a Hill coefficient, $n = 1.7 \pm 0.2$ (Figure 4-1B). This Hill coefficient suggests cooperativity in cobalt binding to YjiA, a surprising result given that the protein binds only one equivalent of cobalt. This discrepancy may be explained by the ability of cobalt to induce oligomerization in the protein (see below). The titration of YjiA with Ni(II) is more complicated due to the 2:1 Ni(II):YjiA stoichiometry since the titration may be monitoring either a single site or both Ni(II) sites simultaneously. The electronic absorption spectrum demonstrates that at least one site is spectroscopically active, but the mutants described below suggest that the second Ni(II) site does not yield a discernable electronic absorption signal. The appearance of a LMCT upon the addition of sub-stoichiometric amounts of nickel to YjiA indicates that the second site is not significantly tighter than the spectroscopically active site. Thus, the second nickel site is either of a similar or weaker affinity than the spectroscopically active site. If this site is much weaker, then the nickel titration in Figure 4-1B represents only the spectroscopically active site and yields an apparent $K_d = (3.9 \pm 0.3) \times 10^{-6}$ M, $n = 1.2 \pm 0.3$ (solid line) (see text). Likewise, Co(II) binding to 5 μM YjiA can be monitored at 350 nm (squares) and fit to yield an apparent $K_d$ of $(2.0 \pm 0.1) \times 10^{-5}$ M, $n = 1.7 \pm 0.2$ (dashed line).

Figure 4-1. Cobalt and nickel binding to WT YjiA. (A) The difference spectra of 20 μM YjiA incubated with 20 μM NiCl₂ (solid line) or 20 μM CoSO₄ (dotted line) display ligand-to-metal charge transfer (LMCT) signals in the region of 250-400 nm. The extinction coefficient was calculated based on the protein concentration. (B) In order to determine the affinity of apo-WT YjiA for Ni(II), the metal was titrated into a sample of 250 nM YjiA and the increase in absorption at 340 nm was used to calculate the fractional saturation of the protein (circles). Data such as those shown were fit to the Hill Equation to yield an apparent $K_d$ of $(3.7 \pm 0.3) \times 10^{-6}$ M, $n = 1.2 \pm 0.3$ (solid line) (see text). Likewise, Co(II) binding to 5 μM YjiA can be monitored at 350 nm (squares) and fit to yield an apparent $K_d$ of $(2.0 \pm 0.1) \times 10^{-5}$ M, $n = 1.7 \pm 0.2$ (dashed line).
0.3) \times 10^{-6} \text{ M} with a Hill coefficient, \( n = 1.2 \pm 0.2 \) for this one site. If the two sites are of similar affinity, an apparent average \( K_d \) for the two sites can be calculated from the Ni(II) titration to be \((3.7 \pm 0.3) \times 10^{-6} \text{ M}, n = 1.2 \pm 0.3\).

Due to the spectroscopically quiet nature of Zn(II), a metallochromic indicator, zincon, was used to estimate the affinity of Zn(II) binding to YjiA. The \( K_d \) of zincon for Zn(II) was determined to be \(10 \pm 1 \mu\text{M} \) under our buffer conditions (data not shown), in agreement with the reported \( K_d \) of \(12.7 \mu\text{M}\).\textsuperscript{23,24} In the absence of protein, metal loading of 140 \( \mu\text{M} \) zincon is complete upon the addition of 140 \( \mu\text{M} \) Zn(II) (Figure 4-2). Upon the inclusion of 10 \( \mu\text{M} \) YjiA, 180 \( \mu\text{M} \) Zn(II) is required to observe saturation of the zincon (Figure 4-2), demonstrating competition by YjiA for Zn(II) and consistent with the measured Zn(II) stoichiometry of four ions per monomer. Furthermore, Zn(II) binding to zincon is not observed until more than 20 \( \mu\text{M} \) metal has been added, suggesting that YjiA has two Zn(II) sites with a \( K_d \) significantly tighter than that of

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure_4-2.png}
\caption{Zinc binding to YjiA. Upon binding Zn(II), the maximum absorption of zincon shifts from 488 nm to 620 nm. It takes 140 \( \mu\text{M} \) ZnSO\(_4\) to saturate 140 \( \mu\text{M} \) zincon (circles), consistent with a 1:1 stoichiometry. In a competition between 140 \( \mu\text{M} \) zincon and 10 \( \mu\text{M} \) apo-YjiA (squares), the spectrum of zincon does not change until after the addition of 20 \( \mu\text{M} \) ZnSO\(_4\), suggesting that there are two Zn(II) sites on YjiA that can outcompete zincon. It takes an additional 40 \( \mu\text{M} \) ZnSO\(_4\) to saturate the 620 nm signal, suggesting that there are two additional sites in YjiA capable of competing with zincon for Zn(II). In the case of the E37A,C66A,C67A mutant (triangles), the initial plateau region is not observed, but 180 \( \mu\text{M} \) ZnSO\(_4\) is still required to saturate zincon, suggesting that the mutant YjiA can still bind 4 Zn(II) ions but with weaker affinities than WT protein.}
\end{figure}
zincon ($K_d < 10 \mu M$), and that the remaining 2 Zn(II) sites have affinities comparable to zincon ($K_d \sim 10 \mu M$). Finally, to confirm the Zn(II) stoichiometry, ESI-MS of YjiA incubated with excess Zn(II) was performed (Figure 4-3). The mass spectrum revealed up to 4 Zn(II) ions bound to the protein, with two sites completely filled and two partially filled, consistent with the affinities of the sites estimated in the competition experiment.

**Ni(II), Co(II), and Zn(II) share a common site.** To determine if the binding sites of the different transition metals overlap, apo-YjiA was incubated simultaneously with two different metals, followed by HPLC metal analysis to identify which metals are bound. When YjiA was incubated with both CoSO$_4$ and NiCl$_2$, $1.9 \pm 0.3$ Ni(II) and $0.15 \pm 0.01$ Co(II) were detected.

![Electrospray ionization mass spectrum of WT YjiA.](image)

**Figure 4-3.** Electrospray ionization mass spectrum of WT YjiA. Observed mass spectrum (top spectrum) and reconstructed masses (bottom spectrum) of WT YjiA after incubating with 5 equivalents of metal. WT YjiA is observed binding two (35786 Da), three (35849 Da) and four (35912 Da) zinc ions. Also observed are the sodiated (+22 Da) and potassiated (+28 Da) peaks for each species.
bound to the protein, suggesting that Co(II) shares a site with Ni(II) and that Ni(II) can outcompete Co(II) for this site. Similar experiments conducted with ZnSO$_4$ and either NiCl$_2$ or CoSO$_4$ demonstrated selectivity for Zn(II) as 3.6 ± 0.3 Zn(II) and 4.0 ± 0.4 Zn(II) per YjiA were detected, respectively, with no detectable Ni(II) or Co(II)).

The GTPase domain contains the common metal-binding site. A sequence alignment of YjiA with other G3E GTPases demonstrates the presence of conserved putative metal-binding residues between the Walker A and Walker B motifs (Figure 4-4A). In the COG0523 subfamily, this conserved sequence corresponds to C$_{64}$XCC$_{67}$ of YjiA. Furthermore, examination of the published crystal structure of apo-YjiA$^{17}$ revealed two nearby glutamates, Glu37 and Glu42, which could also serve as metal-binding residues (Figure 4-4B). To investigate the role of these residues...

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**Figure 4-4. Structure of WT YjiA and location of the metal-binding site in the primary structure of the GTPase domain.** (A) Sequence alignment of the GTPase domain regions between the Walker A and Walker B motifs of representative G3E GTPases generated by the COBALT sequence alignment program (available online at [http://www.ncbi.nlm.nih.gov/tools/cobalt/](http://www.ncbi.nlm.nih.gov/tools/cobalt/)). Located between the Walker A and B motifs is a putative metal-binding motif, the location of which is common among the G3E GTPases. The two glutamates mutated in this study are highlighted by orange boxes. The species from which the protein sequences were derived and the starting sequence position (in brackets) are as follows: YjiA- *Escherichia coli* (11); YeiR- *E. coli* (9); CobW- *Pseudomonas denitrificans* (18); YciC- *Bacillus subtilis* (11); Nha3- *Rhodococcus sp. N-771* (13); UreG- *Klebsiella aerogenes* (14); EcHypB- *E. coli* (111); HpHypB- *Helicobacter pylori* (53). (B) The structure of apo-YjiA (PDB 1NIJ), previously published$^{17}$ features two domains. The N-terminal GTPase domain possesses a typical G3E GTPase fold with a central β-sheet core surrounded by α-helices. Located on one of the central β-strands is the conserved C$_{64}$XCC$_{67}$ motif. Glu37 and Glu42 are near this motif in the folded protein (inset).
glutamates as well as the CXCC motif in metal binding, we generated the E37A,C66A,C67A and the E42A,C66A,C67A triple mutants of YjiA and characterized their metal-binding capabilities. Upon addition of Ni(II) or Co(II) to either triple mutant protein, the absorbance spectra lacked the LMCTs at 340 and 350 nm, respectively, suggesting that Cys66 and/or Cys67 are the source of these signals (data not shown). Furthermore, both triple mutants exhibited diminished Co(II) and Ni(II) binding, but the Zn(II) stoichiometry was unchanged (Table 4-4). However, the previous observation that Zn(II) competes with the other metals, suggesting overlapping sites, led us to further investigate the Zn(II) coordination of E37A,C66A,C67A YjiA by using a zincon competition. Unlike the WT protein, no initial plateau region was observed when the triple mutant was included in the zincon titration, yet 180 µM Zn(II) was still required to saturate 140 µM zincon (Figure 4-2). This result is consistent with the maintained Zn(II) stoichiometry of the mutant protein, but indicates that the mutations have significantly weakened the affinities of two of the metal ions, and suggests that there is at least one Zn(II)-binding site involving some combination of E37, C66, and C67 that has a \( K_d < 10 \) µM. Unlike WT YjiA (Figure 4-3), the metalation state of the zinc-loaded mutant protein could not be observed by mass spectrometry, due to poor signal quality. Upon addition of zinc to the mutant protein, the decrease in signal was more than that observed for WT, suggesting that it cannot just be attributed to ion suppression, and is likely due to changes in the charge state of the mutant protein. Altogether, these experiments indicate that the Ni(II)/Co(II) sites and at least one Zn(II) site contain some combination of E37, E42, C66, and C67 as ligands.

**Metal binding induces oligomerization of YjiA with no major secondary structure changes.** The CD spectrum of apo-WT YjiA (Figure 4-5) is indicative of a mixed αβ protein, in close agreement with the published crystal structure. Upon addition of Co(II), Zn(II), or Ni(II), minor changes in the CD spectrum were detected, but the calculated percentage of secondary structure content was unchanged (Figure 4-5), indicating that YjiA does not undergo any dramatic secondary structure changes upon binding metal.
Analytical gel filtration chromatography was used to probe the oligomeric state of apo-YjiA, as well as the metal- and nucleotide-bound species. In the apo form, the protein elutes from the column as a monomer (Figure 4-6 and Table 4-5). The calculated molecular mass of the monomeric apo-YjiA species from the gel filtration experiments (30.0 ± 1.5 kDa) is smaller than the predicted molecular weight of 35.7 kDa, suggesting a compact protein structure. Upon the addition of 2 equivalents of Ni(II), Co(II), or Zn(II), the protein oligomerizes, forming a small amount of dimer as well as a larger oligomeric species (Figure 4-6). The protein also dimerizes in the presence of GDP or GTP, but to a lesser degree than with metal (Figure 4-6). Some oligomerization was also observed upon adding metal to the E37A,C66A,C67A and E42A,C66A,C67A mutants, although the impact on quaternary structure is diminished compared to the WT protein (Table 4-5).

**Figure 4-5.** Circular dichroism (CD) spectra of WT YjiA and two YjiA triple mutants. (Left) The CD spectrum of 20 μM apo-WT YjiA (red squares) possesses the general characteristics consistent with a mixed αβ protein, in agreement with the published crystal structure of YjiA. Analysis of the spectrum using the K2D3 program indicates the presence of 22% α-helix and 26% β-sheet. Upon the addition of 100 μM NiCl₂ (green circles), 100 μM ZnSO₄ (blue triangles), or 200 μM CoSO₄ (orange diamonds), an increase in negative ellipticity near 208 nm is observed, suggesting a conformational change in the protein. However, K2D3 analysis did not indicate a major change in the overall content of α-helix and β-sheet (for + Ni(II), 23% α-helix, 25.0% β-sheet; for + Zn(II), 22% α-helix, 25.0% β-sheet; for + Co(II), 21% α-helix, 26.0% β-sheet). (Right) CD Spectra of WT YjiA (red squares) compared to E37A,C66A,C66A YjiA (blue triangles) and E42A,C66A,C67A YjiA (green circles).
Metal binding affects YjiA GTPase activity. YjiA was previously shown to bind GTP and based on its nucleotide recognition motif, is predicted to possess GTP hydrolysis (GTPase) activity.\(^{17}\) In agreement with this prediction, the apo-WT protein has low GTPase activity, with a \(k_{cat}/K_m = 14 \pm 9 \text{ M}^{-1} \text{s}^{-1}\) (Table 4-6). To determine whether metal binding modulates the GTPase activity, apo-YjiA was incubated with Ni(II), Co(II), or Zn(II) prior to analysis. The addition of Co(II) slightly disrupts GTPase activity, reducing the \(k_{cat}/K_m\) to \(2.3 \pm 0.8 \text{ M}^{-1} \text{s}^{-1}\). The presence of Ni(II) significantly diminishes the GTPase activity of YjiA, such that substrate saturation was not observed with up to 950 \(\mu\text{M}\) GTP, indicating a significantly weaker \(K_m\) versus that of the apo protein. Attempts to use higher GTP concentrations were unsuccessful due to departure from the linear response region of the assay (data not shown). Zn(II) also inhibits the enzyme, and in this case no activity was detectable in the presence of 100 \(\mu\text{M}\) ZnSO\(_4\) (Table 4-6). The results of these GTPase assays prompted experiments to examine if there was a connection between GDP binding and metal binding to YjiA. Inclusion of 1.5 mM GDP in the protein buffer lowered the metal stoichiometry of all three metals (Table 4-4).

**Figure 4-6. Effect of metal on the quaternary structure of YjiA.** In the absence of metal, 60 \(\mu\text{M}\) YjiA elutes at a volume corresponding to a monomer (solid black line). The addition of 2 equivalents of NiCl\(_2\) (solid red line), CoSO\(_4\) (dashed black line), or ZnSO\(_4\) (solid blue line) results in the formation of dimeric and oligomeric species. Incubation of YjiA with 2 equivalents of GDP (dashed red line) or GTP (dashed blue line) followed by chromatography with 400 \(\mu\text{M}\) nucleotide in the running buffer, resulted in only a small portion of dimeric protein. The chromatographic traces (monitored at 280 nm) are representative datasets from experiments with a Superdex 200 10/300 analytical column equilibrated with 25 mM HEPES, pH 7.6, 200 mM NaCl, and 5 mM MgCl\(_2\). The ticks at the top of the graph denote the elution volumes of the protein standards. From left to right, the identity of the standards and their respective molecular masses are as follows: thyroglobulin (670 kDa), \(\gamma\)-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B\(_{12}\) (1.4 kDa).
Table 4-5: Summary of gel filtration chromatography results of WT and mutant YjiA with added metals and nucleotide.\(^a\)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Added Metal/ Nucleotide</th>
<th>Calculated MW (kDa)</th>
<th>Relative Peak Area(^b)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT YjiA</strong></td>
<td>Apo</td>
<td>30 ± 2</td>
<td>100</td>
<td>Monomer</td>
</tr>
<tr>
<td></td>
<td>2 eq. Ni(II)</td>
<td>31.2 ± 0.4</td>
<td>21.5</td>
<td>Monomer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>316 ± 5</td>
<td>78.5</td>
<td>Oligomer</td>
</tr>
<tr>
<td></td>
<td>2 eq. Zn(II)</td>
<td>38.4 ± 0.7</td>
<td>11.0</td>
<td>Monomer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>440 ± 40</td>
<td>89.0</td>
<td>Oligomer</td>
</tr>
<tr>
<td></td>
<td>2 eq. Co(II)</td>
<td>36.5 ± 0.5</td>
<td>14.5</td>
<td>Monomer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>350 ± 10</td>
<td>85.5</td>
<td>Oligomer</td>
</tr>
<tr>
<td></td>
<td>2 eq. GDP(^c)</td>
<td>29.1 ± 0.1</td>
<td>91.0</td>
<td>Monomer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>62 ± 2</td>
<td>9.0</td>
<td>Dimer</td>
</tr>
<tr>
<td></td>
<td>2 eq. GTP(^c)</td>
<td>28.6 ± 0.1</td>
<td>92.0</td>
<td>Monomer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>61.3 ± 0.8</td>
<td>8.0</td>
<td>Dimer</td>
</tr>
<tr>
<td><strong>E37A,C66A,C67A YjiA</strong></td>
<td>Apo</td>
<td>29.2 ± 0.2</td>
<td>100</td>
<td>Monomer</td>
</tr>
<tr>
<td></td>
<td>2 eq. Ni(II)</td>
<td>29.2 ± 0.8</td>
<td>92.5</td>
<td>Monomer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>63 ± 2</td>
<td>7.5</td>
<td>Dimer</td>
</tr>
<tr>
<td></td>
<td>2 eq. Zn(II)</td>
<td>29.6 ± 0.2</td>
<td>31.0</td>
<td>Monomer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>590 ± 20</td>
<td>69.0</td>
<td>Oligomer</td>
</tr>
<tr>
<td></td>
<td>2 eq. Co(II)</td>
<td>27.7 ± 0.6</td>
<td>100</td>
<td>Monomer</td>
</tr>
<tr>
<td><strong>E42A,C66A,C67A YjiA</strong></td>
<td>Apo</td>
<td>29.7 ± 0.4</td>
<td>94.0</td>
<td>Monomer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>66 ± 1</td>
<td>6.0</td>
<td>Dimer</td>
</tr>
<tr>
<td></td>
<td>2 eq. Ni(II)</td>
<td>29.48 ± 0.02</td>
<td>29.5</td>
<td>Monomer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>600 ± 50</td>
<td>70.5</td>
<td>Oligomer</td>
</tr>
<tr>
<td></td>
<td>2 eq. Zn(II)</td>
<td>29.5 ± 0.1</td>
<td>90.0</td>
<td>Monomer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>66.4 ± 0.2</td>
<td>10.0</td>
<td>Dimer</td>
</tr>
<tr>
<td></td>
<td>2 eq. Co(II)</td>
<td>29.22 ± 0.03</td>
<td>70.0</td>
<td>Monomer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>286 ± 7</td>
<td>30.0</td>
<td>Oligomer</td>
</tr>
</tbody>
</table>

\(^a\)Errors are standard deviations of at least two replicates. The expected molecular weights are: WT YjiA monomer: 35.7 kDa; WT YjiA dimer: 71.3 kDa; WT YjiA tetramer: 142.6 kDa; E37A,C66A,C67A and E42A,C66A,C67A YjiA monomer: 35.5 kDa; E37A,C66A,C67A and E42A,C66A,C67A YjiA dimer: 71.1 kDa; E37A,C66A,C67A and E42A,C66A,C67A YjiA tetramer: 142.2 kDa. In cases where the dimeric and oligomeric species were not well resolved they are summarized as “oligomer”.

\(^b\)The relative peak areas varied by < 4.3 % of the total peak area.

\(^c\)Samples contained 400 µM GDP or GTP in running buffer.
The GTPase activity of the apo-form of the E37A,C66A,C67A mutant is comparable to that of the WT protein, with a $k_{cat}/K_m = 6 \pm 3 \text{ M}^{-1} \text{ s}^{-1}$ (Table 4-6), but unlike WT YjiA, the mutant protein was not inhibited by Ni(II), and Co(II) increased the overall catalytic efficiency of the enzyme to a $k_{cat}/K_m = 78 \pm 9 \text{ M}^{-1} \text{ s}^{-1}$ (Table 4-6). In contrast, addition of Zn(II) to the triple mutant still inhibited GTPase activity, even though it was added at lower concentrations than Co(II) and Ni(II). While the E37A,C66A,C67A triple mutant has reduced affinity for all three metals (see above), it appears that Zn(II) binds to at least some sites in YjiA with higher affinity than Co(II) and Ni(II) do, and that this affinity remains high enough to cause inhibition at the concentrations used. To probe whether this inhibition of GTPase activity occurs by direct Zn(II) binding to the active site or in an allosteric fashion by Zn(II) binding to other metal sites on YjiA, we carried out a structural analysis of Zn(II)-bound YjiA.

### Table 4-6. Kinetics of GTP hydrolysis by WT and E37A,C66A,C67A YjiA

<table>
<thead>
<tr>
<th>YjiA Variant</th>
<th>Metal Bound</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (M)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Apo</td>
<td></td>
<td>$(6 \pm 2) \times 10^{-4}$</td>
<td>$(5 \pm 3) \times 10^{-3}$</td>
<td>$14 \pm 9$</td>
</tr>
<tr>
<td>Co(II)</td>
<td></td>
<td>$(5 \pm 2) \times 10^{-4}$</td>
<td>$(2.3 \pm 0.7) \times 10^{-4}$</td>
<td>$2.3 \pm 0.8$</td>
</tr>
<tr>
<td>Ni(II) WH</td>
<td></td>
<td>No Measurable Hydrolysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn(II)</td>
<td></td>
<td>No Measurable Hydrolysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E37A,C66A,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C67A Apo</td>
<td></td>
<td>$(5 \pm 1) \times 10^{-4}$</td>
<td>$(1.0 \pm 0.7) \times 10^{-4}$</td>
<td>$6 \pm 3$</td>
</tr>
<tr>
<td>Co(II)</td>
<td></td>
<td>$(1.1 \pm 0.1) \times 10^{-3}$</td>
<td>$(1.7 \pm 0.1) \times 10^{-4}$</td>
<td>$78 \pm 9$</td>
</tr>
<tr>
<td>Ni(II)</td>
<td></td>
<td>$(6 \pm 1) \times 10^{-4}$</td>
<td>$(3 \pm 1) \times 10^{-4}$</td>
<td>$2 \pm 1$</td>
</tr>
<tr>
<td>Zn(II)</td>
<td></td>
<td>No Measurable Hydrolysis</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*aAll GTPase assays were conducted with 0.5 – 2 µM WT or E37A,C66A,C67A YjiA in protein buffer supplemented with 5 mM MgCl$_2$. Samples containing metal were preincubated with either zinc, cobalt, or nickel overnight at 4 °C in an anaerobic glove box. The amount of released phosphate was detected using a modified Malachite Green assay. The buffer contained 100 µM Zn(II), 100 µM NiCl$_2$, or 250 µM CoSO$_4$ for WT or 225 µM Zn(II), 400 µM NiCl$_2$, or 800 µM CoSO$_4$ for the E37A,C66A,C67A triple mutant. The data listed are average values from at least three independent experiments. WH, weak hydrolysis (see main text for details)."
**Zn(II) binding induces a space group transition in YjiA crystals.** We determined the crystal structure of Zn(II)-bound WT YjiA to 2.57 Å resolution after soaking apo-WT YjiA crystals with ZnSO₄ (Figure 4-7). Surprisingly, the crystals underwent a space group transition upon soaking. While unsoaked (apo) WT YjiA crystals belong to space group \( C\) with one protomer in the asymmetric unit, \(^{17}\) the space group is \( P_{21} \) after soaking and features two protomers in the asymmetric unit (for a more detailed discussion of space group assignment, see reference \(^{50}\)). The arrangement of molecules in the crystal lattices of Zn(II)-soaked WT YjiA and of apo WT YjiA, however, is nearly identical despite the different space groups (Figure 4-8). It seems that Zn(II) soaking induced a small shift in the crystal lattice, causing a reduction in symmetry from \( C_2 \) to \( P_{21} \) while leaving the overall crystal lattice intact.

Unfortunately, we have not been able to generate nucleotide-bound structures of YjiA. While YjiA exhibits the canonical GTPase motifs, these motifs are not well ordered in the structure and the phosphate-binding Walker A motif in particular seems to exhibit multiple conformations (Figure 4-9). Likely, nucleotide binding will result in a conformational change and depend on the presence of an effector protein, consistent with our unsuccessful attempts to generate nucleotide-bound structures.

**YjiA binds Zn(II) in four distinct sites.** Analysis of the electron density and of Zn anomalous difference Fourier maps revealed the presence of Zn(II) ions in four distinct types of sites: a “bridging site” that is located at the interface of the two YjiA protomers in the asymmetric unit (site B in Figure 4-7), an “internal site” (site C in Figure 4-7), and two types of “surface sites” (sites D and E in Figure 4-7). In total, the two YjiA protomers in the asymmetric unit could bind seven Zn(II) ions: two in the internal sites, one in the bridging site, and four in surface sites (Figure 4-7). The asymmetric unit, however, only contains five Zn(II) ions because all four surface sites are involved in crystal lattice contacts and each of those bound Zn(II) ions is shared with a neighboring asymmetric unit molecule.
**Figure 4-7. Crystal structure of Zn(II)-bound WT YjiA.** (A) Overall structure of the two YjiA protomers in the asymmetric unit (yellow and green ribbons). Symmetry-related molecules involved in Zn(II) binding are shown as gray ribbons. Four types of Zn(II)-binding sites are observed in the structure: a bridging site (labeled B), an internal site (labeled C), and two types of surface sites (labeled D and E). Bound Zn(II) ions are shown as purple spheres and coordinating residues are shown as sticks. A Zn anomalous difference Fourier map is contoured around bound Zn(II) ions at 5 σ. (B) Close-up view of the bridging Zn(II)-binding site, located on a two-fold axis between the two protomers in the asymmetric unit. Zn(II) is coordinated by E74 and H114 from both protomers. (C) Close-up view of the internal Zn(II)-binding site. The side chains of E37, E42, and C66 (green carbons) coordinate the Zn(II), with an open coordination sphere probably occupied by a water molecule. The structure of apo-WT YjiA (PDB ID 2NIJ) is superimposed and shown with magenta carbons. The region around the Zn(II)-binding site undergoes conformational changes upon Zn(II) binding, as indicated by the arrows. The Zn(II)-binding site is also located in close proximity to the nucleotide binding site, as demonstrated by modeling studies with a GTP-analog-bound HypB structure (PDB ID 2HF8, GTPγS shown with cyan carbons and Mg(II) shown as an orange sphere). (D) and (E) Close-up views of the two types of surface sites, located at crystal contacts between YjiA protomers in the asymmetric unit and crystallographically related molecules. Zn(II) is coordinated by the side chains of E167 and/or H170 from one molecule and H187 from the other molecule.
The internal site is located near the GTPase active site. The internal site is located in the N-terminal region of a YjiA protomer, with the side chains of E37, E42, and C66 coordinating Zn(II) (Figure 4-7C). Although hard to resolve in the electron density at this resolution, a fourth coordination site on Zn(II) could be occupied by a water molecule, which would give rise to a tetrahedral coordination geometry. E37 and E42 are located in a loop region that undergoes a conformational change upon Zn(II) binding to bring the side chain of E42 within proximity of the bound Zn(II) (Figure 4-7C). Furthermore, the region around C66 undergoes a slight shift, bringing C66 closer to the Zn(II) ion and re-arranging the subsequent loop region significantly (Figure 4-7C).

It is clear that the internal Zn(II)-binding site is in the neighborhood of the nucleotide-binding site (Figure 4-7C), as demonstrated by a structural alignment with the GTP-analog-bound structure of *Methanocaldococcus jannaschii* HypB (PDB ID 2HF8\(^{18}\)), which has the highest

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**Figure 4-8.** Comparison of crystal lattices of apo-WT YjiA (blue, PDB ID 1NIJ\(^{17}\)) and Zn(II)-soaked WT YjiA (asymmetric unit in yellow and green, remaining protomers in gray). The crystal lattices of the two structures are almost identical.
structural homology with YjiA of the proteins in the protein data bank (Cα r.m.s.d. of 3.1 Å over 209 residues, determined by DaliLite52). In this model, the nucleotide-associated Mg(II) is within 5.4 Å of the side chain carboxylate of E37 and within 5.3 Å of the Cβ of E39 (Figure 4-10), which has a disordered side chain. These observations raise the possibility that E37 or E39 are involved in Mg(II) coordination upon nucleotide binding. Notably, E39 is the first residue of the switch I motif of YjiA, a common NTPase motif known to undergo conformational changes during NTPase activity, as indicated by structural and sequence alignment (Figure 4-4A). In M. jannaschii HypB, the first residue of the switch I motif, D75, is one of the ligands to the nucleotide-associated Mg(II) (Figure 4-10).18 In our structural alignment, D75 of M. jannaschii HypB aligns with E39 of YjiA (Figure 4-10), further suggesting that E39 could be involved in Mg(II) coordination.

The structure of this internal site is consistent with the solution studies of metal binding to WT and mutant YjiA. Given that mutagenesis of some of these ligands disrupts Co(II) and Ni(II) binding, it is likely that a variation of this internal site also binds these other metals in addition to
Zn(II), with the coordination by C66 producing the LMCT absorption features observed for the Co(II)- or Ni(II)-loaded protein. Furthermore, the observation that Zn(II) can outcompete both Ni(II) and Co(II) for binding suggests that this site is one of the higher-affinity sites observed in the zincon competition assay, in agreement with the weakened affinity of the E37A,C66A,C67A mutant. As the internal site is in close proximity to the GTPase active site and involves the GTPase switch I motif, it is conceivable that the metal-induced inhibition of GTPase activity is due to metal binding at this site. Taken together, these data indicate that the internal site can be occupied by different metals with low micromolar affinity, causing subtle conformational changes that modulate the function of YjiA.

Figure 4-10. E37 or E39 of YjiA could coordinate nucleotide-associated Mg(II). GTP-analog-bound HypB (cyan carbons, PDB ID 2HF818) is superimposed onto the structure of Zn(II)-bound WT YjiA (green carbons). Bound GTPγS is shown with cyan carbons and GTPγS-associated Mg(II) is shown as an orange sphere. D75 of HypB is involved in Mg(II)-coordination. Both E37 and E39 of YjiA are within proximity of the modeled Mg(II), as indicated by the purple dashed lines, raising the possibility that these residues are involved in Mg(II) coordination in YjiA. Notably, E37 is also involved in binding Zn(II) (purple sphere). E39 was truncated to the Cβ atom due to lack of electron density for the side chain.
To further probe metal binding to the internal site, we solved the crystal structure of Zn(II)-bound E37A,C66A,C67A YjiA to 2.05 Å resolution. The overall structures of WT YjiA and E37A,C66A,C67A YjiA are nearly identical, with a Cα r.m.s.d. of 0.450 Å between the two structures (Figure 4-11A). While the bridging site and the surface sites are still occupied with Zn(II), the internal site lacks bound Zn(II) as indicated by both the electron density and the Zn anomalous difference Fourier map (Figures 4-11B, 4-11E). Instead, it appears that the side chain of F40 has moved into the space previously occupied by the side chains of C66 and E37 as well as the Zn(II) ion (Figure 4-11C).

**The bridging site and the surface site connect YjiA protomers.** As mentioned briefly above, the bridging site is located between two YjiA protomers on a non-crystallographic two-fold symmetry axis, with Zn(II) coordinated by the side chains of E74 and H114 from both protomers in approximate tetrahedral geometry (Figure 4-7B). Binding to this site likely disrupted the C2 symmetry of apo-WT YjiA crystals, as the bridging site would be located on a crystallographic axis in the C2 lattice. Analysis of the resulting dimer interface with the PISA server\(^5^3\) indicates that this interface alone is not as extensive as is typically observed in physiologically relevant dimers, with an average buried surface area of 1244 Å\(^2\) and a low complex formation significance score of 0.132 (on a scale of 0 to 1 with increasing relevance to complex formation in solution), not including the contributions from Zn(II). Thus, it seems unlikely that apo-YjiA would be a dimer in solution, in agreement with our analytical gel filtration chromatography results. Zn(II) binding, however, could strengthen this interface and thereby induce dimerization and oligomerization of YjiA.
Figure 4-11. Crystal structure of Zn(II)-soaked E37A,C66A,C67A YjiA. (A) Overall structure of the two E37A,C66A,C67A YjiA protomers in the asymmetric unit, colored in pink and orange ribbons. The crystal structure of Zn(II)-soaked WT YjiA is superimposed (coloring as in Figure 4-7). (B) Close-up view of the bridging Zn(II)-binding site. This site is still occupied with Zn(II) (purple sphere), as indicated by the Zn anomalous difference Fourier map contoured at 5 σ (yellow mesh). Zn(II)-coordinating residues are labeled. (C) Comparison of the internal sites of Zn(II)-soaked WT YjiA (green carbons) and Zn(II)-soaked E37A,C66A,C67A YjiA (pink carbons). For the triple mutant, F40 occupies the space left open by the mutations and the lack of Zn(II). A 2F_o–F_c simulated annealing composite omit map (blue mesh) is contoured around F40 at 1.0 σ. The side chain of E42 is truncated to the Cβ in E37A,C66A,C67A YjiA due to lack of electron density. (D) and (E) Close-up views of the surface Zn(II)-binding sites. Crystallographically related YjiA molecules are shown with gray carbons. Map and coloring as in (B).
All four surface Zn(II) sites are located at crystal contacts at the interfaces of YjiA protomers (Figures 4-7D, 4-7E). The Zn(II) ions in these sites are coordinated by H170 and/or E167 from one protomer and H187 from a protomer in the neighboring asymmetric unit, or vice versa. These sites exhibit a significant amount of disorder and high crystallographic B-factors, indicating that the Zn(II) ions are not bound at high occupancy within the crystal lattice. There are also some differences in the exact coordination spheres of the surface sites, providing further rationale for the asymmetry that led to the space group transition upon Zn(II) binding. Most likely, Zn(II) binding affects the individual protomers in a slightly different manner, thus causing breakdown of the crystallographic symmetry. It is unclear whether these sites would be occupied by Zn(II) ions in solution or whether binding to these sites occurred adventitiously, after the protein ligands were brought into proximity by crystallization. These sites could, however, provide an additional explanation for the observed metal-induced oligomerization of YjiA, as they could stabilize higher order oligomers.

4.4 Discussion

The mechanisms through which cells ensure the proper allocation and trafficking of metal ions are not well understood. The COG0523 subfamily of GTPases represents a large and diverse group of proteins and, based on homology to known metallochaperones in the other G3E subfamilies and comparative genomics analysis, it was suggested that they play a role in metal trafficking or some other metal homeostasis process.\textsuperscript{6,54} This study of YjiA lends credence to this proposal. In particular, we demonstrate that YjiA can bind transition metals and provide biochemical and structural details about this interaction. Furthermore, the impact of metal binding on the GTPase activity of YjiA suggests that metal ions play a regulatory function.

YjiA belongs to subgroup 9 of the COG0523 subfamily and was predicted to bind metals via a conserved CXCC motif.\textsuperscript{17} As with the other members of this subgroup, YjiA does not have an assigned function, but there is a growing body of evidence that suggests a role for YjiA in the cellular response to carbon starvation.\textsuperscript{6,55-57} Within the genomic context, \textit{yjiA} is found downstream of \textit{yjiY}, a homolog of the carbon starvation protein CstA, and \textit{yjiX}, a small cytosolic protein of unknown function.\textsuperscript{6} These three genes are transcribed as a single transcript,\textsuperscript{56} which is believed to be under the control of one of the central regulators in the carbon catabolism pathway, cyclic AMP receptor protein (CRP).\textsuperscript{57} Furthermore, \textit{yjiA} is down-regulated by the non-
coding RNA molecule Spot 42 that participates in a feed-forward loop to help enact catabolite repression in *E. coli*. Further studies will be needed to establish what role YjiA plays in the carbon starvation response and the function of metals in this pathway.

Here, we present a characterization of the metal-binding capabilities of YjiA. In particular, our data identify a Zn(II)-binding site created by the side chains of E37, E42, and C66, the latter from the predicted C64XCC67 metal-binding motif. Solution studies of wild type and mutant proteins suggest that Ni(II) and Co(II) also bind to at least a subset of the same ligands, although the coordination is likely to be different. Whether or not the other conserved cysteines in this motif have a functional role in binding metals or performing downstream effects remains to be determined. The relative metal affinities mirror the Irving-Williams series of small-molecule chelators for divalent metal ions, suggesting that the protein structure is not enforcing strong metal selectivity. Furthermore, at this time, it is not clear which metals are physiologically relevant for YjiA function. Many of the COG0523 subfamilies were linked to zinc pathways due to the presence of zinc-regulated genetic elements, and deletions of *B. subtilis yciC* or *E. coli yeiR* sensitize the bacteria to zinc-deficient conditions. However, a similar phenotype was not observed in a ΔyjiA strain of *E. coli* and binding sites for zinc metalloregulators were not detected in the genetic neighborhoods of subgroup 9. On the other hand, although nickel is required in *E. coli* as a cofactor for several [NiFe]-hydrogenase isoforms, the uptake of this metal is restricted to anaerobic growth and there is no evidence that expression of *yjiA* is similarly controlled. Finally, a requirement for cobalt ions has not been identified in *E. coli* and this organism does not make its own cobalamin. It is possible, however, that the metal-binding capabilities of YjiA are only called upon to meet the challenges of unusual growth conditions.

The internal metal-binding site is adjacent to the GTPase active site, as predicted by our nucleotide modeling studies (Figures 4-7C, 4-10). Most notably, the region involved in metal binding contains the switch I motif (Figure 4-4A), raising the possibility that metal binding restricts the conformational changes of this motif, an essential part of the GTPase cycle. In addition, structural homology suggests that E39 and E37 are candidates for Mg(II) coordination, and the ability to coordinate Mg(II) could be affected by the presence of metals in the internal site. Indeed, we observe that the presence of metals affects the GTPase activity of YjiA, with Zn(II), Co(II), and Ni(II) suppressing GTPase activity to different extents, and that GDP can modulate metal binding. Given that the CXCC motif is conserved in the COG0523 subfamily, it
is likely that metal-dependent regulation of activity is also conserved. This hypothesis is consistent with a recent study of \textit{E. coli} YeIR,\(^{16}\) although in this latter case the GTP hydrolysis was accelerated in the presence of metals. Perhaps structural differences in the GTPase domain metal-binding sites are responsible for the variations on this theme. YjiA and YeIR are highly homologous and are well conserved within the GTPase domain, including the CXCC motif and Glu42 (YjiA numbering), but Glu37 is not conserved (Figure 4-4A). Metal-responsive GTP hydrolysis was also observed for proteins from the HypB G3E subfamily,\(^{61-63}\) with the most dramatic impact observed with Zn(II). While these proteins share structural similarity to YjiA in their GTPase domain and also have a GTPase domain metal-binding site, there are some clear architectural differences. The YjiA internal metal-binding site is located in a small “pocket” of the enzyme with a solvent-exposed opening, whereas in the \textit{M. jannaschii} HypB structure the metal-binding site is located on the surface of the protein (Figure 4-12).\(^{18}\)

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure412.png}
\caption{Comparison of the metal-binding sites of YjiA and HypB. (A) The internal metal-binding site of YjiA is located in a solvent-accessible pocket of the protein beneath the surface of the protein. YjiA protomers in the asymmetric unit are shown in surface representation in green and yellow, with ribbons shown underneath. Bound Zn(II) in the internal site is shown as a purple sphere, additional Zn(II) ions are shown as gray spheres. The inset shows a close-up view of the internal Zn(II)-binding site. (B) Superimposition of a YjiA protomer and a HypB dimer (PDB ID 2HF8\(^{18}\)), shown in the same orientation as in panel (A). HypB is colored in cyan and gray and HypB-bound Zn(II) ions are shown as orange spheres. Although YjiA and HypB share structural homology in the GTPase domain, the Zn(II)-binding sites of HypB are located on the protein surface and do not resemble the internal Zn(II)-binding site in YjiA. (C) The HypB Zn(II)-binding sites are located directly on the surface of the protein. HypB is colored and oriented as in panel (B).}
\end{figure}
The GTPase activity of YjiA is comparable to those of other G3E GTPases such as *E. coli* HypB, *H. pylori* HypB, and *E. coli* YeiR, none of which feature particularly high hydrolysis rates, suggesting that their in vivo rates may be different from the rates determined in vitro. In vitro, the isolated GTPases lack additional partner proteins and cofactors that may stimulate GTPase activity in vivo. For example, the addition of SlyD, a protein involved in the nickel-insertion stage of [NiFe]-hydrogenase biosynthesis, enhances the GTPase activity of *E. coli* HypB. By analogy, it is possible that YjiA requires an additional factor to maximally activate GTP hydrolysis. A possible candidate is the small cytosolic protein YjiX that is co-transcribed with YjiA.

In addition to the internal metal site, YjiA also binds multiple Zn(II) ions at interface sites between separate YjiA molecules, which may provide an explanation for the metal-induced oligomerization observed by analytical gel filtration chromatography. It is also possible, however, that rearrangements around the internal metal site could create interfaces for oligomerization. Large-scale metal-induced rearrangements would be masked in our crystal soaking experiments because the interfaces between YjiA molecules are already pre-formed in the crystal lattice. This scenario would explain how Co(II) can mediate changes in quaternary structure, given that the solution analysis indicates that only a single Co(II) ion binds to YjiA at the internal site. Furthermore, this model is consistent with the observation that mutation of two of the internal site ligands diminishes oligomerization induced by Ni(II) and Zn(II). Finally, there may be other metal sites on YjiA not identified by crystallography. A recent bioinformatics analysis of the apo-YjiA crystal structure suggested two surface-exposed sites with potential for metal binding, composed of H23, E27, and H29 as well as D52, D79, and D82, respectively. We do not observe bound metal in either of these two sites and E27 and D52 to not appear to be in the correct positions for metal binding. We note, however, that binding to these sites could rely on structural rearrangements that are prohibited by the crystal lattice. Further studies will be necessary to probe the contribution of these residues to metal binding as well as the functional relevance of metal ions bound on the surface of YjiA.

Although characterization of the E37A,C66A,C67A mutant provides information about the structure and impact of the internal Zn(II) site, several observations require additional explanation. The ability of this triple mutant to bind 4 Zn(II) ions and to oligomerize in the presence of Zn(II), albeit to a lesser degree than WT YjiA, suggests that mutation of these
residues is not sufficient to completely disrupt Zn(II) binding. This conclusion, supported by the zincon competition illustrating a decreased affinity for Zn(II), is surprising because the crystal structure depicts a Zn(II) coordination sphere comprised of E37, E42, C66, such that mutation of two of these three protein ligands should be sufficient to stop metal binding. Structural rearrangement of this site to incorporate other residues as ligands, such as the nearby E39, could serve as a possible explanation. Such a rearrangement may not be observed in the E37A,C66A,C67A YjiA crystal structure due to the aforementioned pre-formed interfaces in the crystal prior to Zn(II) binding. Possible binding to a rearranged site in the E37A,C66A,C67A mutant may also explain the GTPase inhibition in the presence of Zn(II). An alternative explanation for the inhibition of the GTPase activity by Zn(II) is the involvement of additional allosteric sites, but evidence to support this latter explanation is needed.

Altogether, the results presented here provide a detailed biochemical and structural picture of the metal-binding properties of a COG0523 GTPase. A connection between members of this subfamily of GTPases and intracellular metal pathways is an emerging trend. This study supports the model that metals play a role in regulating the proteins’ enzymatic activities and offers a glimpse into how this metal-mediated regulation may occur. Furthermore, as noted earlier, subgroup 9 of the COG0523 GTPases is hypothesized to be involved in carbon starvation and the metal-binding activities of YjiA offers a tantalizing hint about the interplay of metal homeostasis with other cellular processes.
4.5 References


5 Summary, Significance, and Future Directions

Although the use of nickel is considered to be inherited from a bygone era when nickel was more plentiful in the ecosystem,\(^1,^2\) it is becoming apparent that this metal still serves a critical role in a variety of biological processes including bacterial pathogenesis\(^3^-^6\) and the global carbon cycle.\(^7,^8\) To date, nickel is a required cofactor for at least ten different enzymes (Chapter 1) in a variety of organisms spanning prokaryotes to fungi and plants. This requirement necessitated the evolution of nickel homeostasis pathways to ensure proper acquisition and control over the metal and to avoid toxic effects of excess nickel.\(^9^-^11\) Progress has been made in understanding these pathways,\(^12^-^14\) but important questions remain, notably on a molecular level. By gaining a better understanding about the coordination spheres, allosteric changes, thermodynamics, kinetic behavior and other molecular-level properties of the proteins involved in nickel homeostasis, a deeper insight into nickel trafficking will be achieved and will contribute vital information to the field of bioinorganic chemistry as a whole. In this thesis, the molecular details of \textit{H. pylori} HypB and a homolog, \textit{E. coli} YjiA, were examined, and depict an intricate system in which metal binding can be used to drive allosteric changes, affecting the overall biochemical properties of the protein.

5.1 The Relationship Between Metal and the Biochemical Properties of \textit{H. pylori} HypB

The biosynthesis of the \textit{H. pylori} [NiFe]-hydrogenase and urease metalloconers requires the participation of multiple dedicated accessory proteins, HypABCDEF and UreIEFGH, respectively. Interestingly, \textit{Hp}HypA and \textit{Hp}HypB are critical for maturation of both [NiFe]-hydrogenase and urease in \textit{H. pylori}, suggesting that these two proteins play a key role in the nickel homeostasis of the organism.\(^15\) Significant work had been conducted on the \textit{Ec}HypB protein,\(^16^-^21\) but several key differences between the two homologs stirred scientific curiosity. For instance, \textit{Hp}HypB lacks \textit{Ec}HypB’s high-affinity metal-binding site found to be indispensable for [NiFe]-hydrogenase maturation in \textit{E. coli}\(^20\) and \textit{Ec}HypB participates only in [NiFe]-hydrogenase maturation whereas \textit{Hp}HypB is also involved in the maturation of urease.\(^15\) The study described in Chapter 2 examined the metal-binding properties of \textit{Hp}HypB and demonstrated a unique link between the metal-binding and biochemical properties of the protein. Based on homology to \textit{Ec}HypB, \textit{Hp}HypB was predicted to bind metal at a CH motif found in the GTPase domain
Metal-binding assays demonstrated that HpHypB can bind stoichiometric nickel or zinc with nanomolar affinity, tighter than EcHypB.\textsuperscript{18} Mutation of the predicted CH ligands suggested that the location of this metal-binding site in the protein is conserved between homologs, and in conjunction with the structural study conducted on \textit{M. jannaschii} HypB,\textsuperscript{22} suggests that it is universally conserved in all HypB proteins. Interestingly, metal binding by HpHypB alters the biochemical properties of the protein, notably the oligomeric and enzymatic activities. This finding is significant because it marked the first report of metal binding in a G3E GTPase acting as an allosteric regulator. Since this initial report, similar allosteric regulation was described in \textit{EcHypB},\textsuperscript{21} \textit{E. coli} YeiR,\textsuperscript{23} and YjiA (Chapter 4). Overall, this investigation established that the metal-binding and biochemical properties of HpHypB are intimately connected and may be important for the function of HpHypB in the maturation of [NiFe]-hydrogenase and urease.

5.2 The Selective Modulation of Metal Coordination by Nucleotide in \textit{H. pylori} HypB

The discovery of a connection between metal binding and the GTPase activity of HpHypB (Chapter 2) prompted an in depth examination of this link on a molecular level. Several questions remained from the initial study, such as the nature of the Ni(II) and Zn(II) coordination spheres, the role of additional cofactors in stimulating GTPase activity, and the allosteric mechanism. The work conducted in Chapter 3 examined these topics and was greatly aided by the first Ni(II)-bound structure of a HypB protein, obtained by our collaborators. This structure suggested that in the GTP- and Ni(II)-bound state, the nickel is bound in a square planar geometry and His107 is no longer a nickel ligand, a finding contrary to previous studies of HypB homologs.\textsuperscript{18,20} Analysis of a site-directed mutant protein suggested that His107 is only a ligand in the absence of nucleotide, in support of the elucidated structure. Furthermore, loading HpHypB with nucleotide was found to alter the Ni(II) stoichiometry and affinity, but surprisingly not that of Zn(II). This finding led to the theory that HpHypB may be able to differentiate between Ni(II) and Zn(II) on the basis of coordination changes upon nucleotide binding, thereby only allowing Ni(II) to be delivered to the nascent [NiFe]-hydrogenase or urease. Under this proposed mechanism, GTP hydrolysis would occur only if Ni(II) was bound and in the GDP-bound state, Ni(II) could then be released.
In addition to the connection between nucleotide binding and metal binding, the allosteric mechanism of *HpHypB* was further elaborated in this study. Cys142 was confirmed to be a metal-ligand and kinetic analysis of a C142S mutant demonstrated that this residue may be involved in transducing the metal-binding event to altered GTPase activity. This conclusion, based on the location of Cys142 within the Switch II motif of *HpHypB*, begins to shed light on the allosterics involved in the protein. However, the study also indicated that additional components may be involved in this allosteric mechanism, such as His107, suggesting that the overall communication between metal- and nucleotide-binding may be more complex. Furthermore, additional factors, such as potassium, were found to be critical for the GTPase activity, demonstrating a need for caution when studying these enzymes in vitro.

### 5.3 The Characterization of a HypB Homolog: *E. coli* YjiA

The connection between metal-binding and biochemical properties of the HypB proteins led to the theory that this link may be a common trait in the G3E GTPases, the family to which HypB belongs.\(^2^4\) These GTPases all feature putative metal-binding motifs located within the same general region of the G-domain, suggesting similar modes of action (Figure 4-4). To further explore this possible family trait, a representative from a different subfamily of G3E GTPases was examined. *E. coli* YjiA was selected for this analysis because a crystal structure of the apo-protein was previously determined\(^2^5\) and this subfamily is largely uncharacterized,\(^2^6\) thus an in depth biochemical study may assist in elucidating a function for these proteins.

The metal-binding characteristics of YjiA are more complex than that of *HpHypB* as the protein can bind multiple Zn(II) or Ni(II) ions. Nonetheless, the study concentrated on the metal site homologous to the one found in HypB, located between the Walker A and B motifs in the G-domain. The architecture of this site is drastically different, consisting of a Cys residue and glutamates, as determined through site-directed mutagenesis and crystallographic studies. Akin to HypB, this site can accommodate multiple types of ions, binding Zn(II), Ni(II), or Co(II), with nanomolar to low micromolar affinities. Furthermore, metal binding induced oligomerization in the protein and altered the GTPase activity, much like the metal-altered biochemical properties of *HpHypB*. Together, these data demonstrate that this link between metal-binding and the biochemical properties of the protein are likely a family-wide trait.
The crystal structure of Zn(II)-bound YjiA allows for the examination of the link between metal binding and GTPase activity. The metal-binding site and GTPase active site are adjacent to each other in the structure, suggesting a direct link between them. It is hypothesized that Zn(II) binding may cause a Mg(II)-coordinating glutamate to be sequestered from the GTPase active site, thereby preventing hydrolysis by removing the ability of the protein to coordinate the catalytically essential Mg(II) ion. In contrast, although no mechanism is clearly evident from the HpHypB structure, it is clear that the mode of action will be distinct from that of YjiA as the metal-binding and GTPase active sites are more distant from one another and metal binding does not appear to alter any residues involved in Mg(II)-coordination. This finding is interesting as it suggests that despite these GTPases comprising a single protein family and featuring similar relative motif positions, their allosteric mechanisms are likely to be different.

5.4 Future Experimental Directions

There are many outstanding questions pertaining to HpHypB which clearly delineate future avenues of research. For example, although the foundation has been laid in this thesis, a complete understanding of the allosteric mechanism in HpHypB is lacking. Future structural and mutagenesis studies would assist in determining the communication between the metal-binding and active sites in HpHypB. Further, such studies should assist in understanding the role of His107 and Cys142 in metal discrimination. Allosterics aside, there are three major directions future research in this area should investigate: the elucidation of the complete coordination sphere, determination of the physiologically relevant metal, and the link afforded by HpHypA and HpHypB between the urease and [NiFe]-hydrogenase pathways.

Although the crystal structure and mutagenesis of HpHypB demonstrated the Ni(II)-coordination sphere in the presence of GTP, the coordination sphere of other nucleotide-bound states remain to be determined, as well as those for Zn(II). X-ray absorption spectroscopy (XAS) would be the ideal technique to study these coordination sphere changes and has already been applied to the study of EcHypB.20 The advantage of this method is that the coordination geometry, ligands, and bond lengths can be obtained from the experiment in the presence of different nucleotides.27-29 More distant extensions of this research direction would be to examine coordination sphere changes in the presence of additional maturation proteins, such as HypA or SlyD.
In the HpHypB studies described in this thesis, both nickel and zinc were examined. To date, it is unclear which metal ion is physiologically relevant. The metal ions alter the protein’s biochemical properties in different ways and both ions are imported for use in *H. pylori*. Although Ni(II) is a logical choice for the physiologically relevant metal given the role of HpHypB in the maturation of two nickel enzymes, there is no clear evidence to support this. In the case of the HypB homolog UreG, responsible for urease maturation, the metal site would also be predicted to bind Ni(II) in vivo given urease’s need for Ni(II), but recent reports suggest that Zn(II) may instead be the relevant ion.\textsuperscript{30,31} The identity of this ion in vivo needs to be addressed to properly understand the role of HpHypB in the maturation of [NiFe]-hydrogenase and urease.

The role of HpHypB and HpHypA in both urease and [NiFe]-hydrogenase maturation is a unique feature of the *Helicobacter* genus and demonstrates a close connection between the two pathways. Recent studies have shed light on this topic,\textsuperscript{32,33} but the role of HpHypB, the reason for this double duty by these two proteins, and the mechanism of control of nickel allocation between the urease and [NiFe]-hydrogenase pathways remain unclear. By understanding this crossroads between these two pathways, a deeper understanding into the nickel homeostasis of the *Helicobacter* genus will be obtained, possibly opening the door for therapeutic treatments against this group of pathogenic bacteria, a topic elaborated on in the next section.

5.5 Significance of the Study of Nickel Bioinorganic Chemistry

Aside from pure academic and scientific merit, there are a growing number of motives driving the study of nickel bioinorganic chemistry in today’s scientific community. For example, biotechnology is embracing [NiFe]-hydrogenases as a means for bioremediation of heavy metals by catalyzing the reduction of heavy metals found in industrial waste.\textsuperscript{34,35} By studying this enzyme and the properties of the catalytic nickel, more efficient and broader bioremediation catalysts can be engineered. Another worthy example is the study of metallocenter assembly pathways in general. In Chapter 3, the studies pertaining to the effect of potassium on HpHypB’s GTPase activity were inspired by studies conducted on the maturation pathway of [FeFe]-hydrogenase.\textsuperscript{36} Although the two pathways feature different accessory proteins and metals, parallels could still be drawn from one system and extrapolated to another. In this way, by studying one system, we can assist in elucidating the aspects of different pathways by highlighting novel aspects or common themes. A third driving force is the current global energy
crisis that is causing new sources of renewable energy to be sought. The [NiFe]-hydrogenases, which can naturally combine electrons and protons to create hydrogen gas, are an attractive mechanism of energy production as they are CO₂-neutral and minimally polluting. The [NiFe]-hydrogenases can be coupled to the sun’s energy using recombinant photosynthetic systems, thereby producing hydrogen gas from the sun’s light and a source of protons. Aside from these examples, the most valuable aspect of studying nickel bioinorganic chemistry is perhaps the potential for the development of new therapeutics.

Nickel is a critical metal for many pathogenic bacteria, such as *E. coli*, *H. pylori*, *Mycobacterium tuberculosis*, *Yersinia pestis*, *Salmonella enterica*, and *Brucella suis*. In these bacteria, nickel plays an essential role through enzymes such as urease and [NiFe]-hydrogenase. Recently, several fungal strains, such as *Cryptococcus neoformans* and *Coccidioides immitis*, have also been found to be dependent on nickel for virulence. With the alarming rate at which microorganisms are acquiring resistance to current therapeutics and the lack of novel antibiotics in development, there is a dire need to develop new ways to attack pathogens. With no known nickel requirement in humans, targeting the nickel homeostasis pathways in these pathogens may be an effective strategy. However, in order to identify appropriate targets, we need to gain a better understanding of the mechanisms of nickel-enzyme biosynthesis and the mechanisms of nickel homeostasis.

Although targeting nickel homeostasis in prokaryotes and fungi may be an attractive therapeutic target, caution will need to be exercised. Nickel depletion studies in rats demonstrated impaired iron absorption, altered lipid metabolism, decreased growth, and altered liver development, even though no metalloenzymes requiring nickel have been identified in vertebrates. These findings suggest that there may be overlooked roles for nickel in vertebrates, or, at the very least, suggest a delicate interplay between various metal ions within biological systems, topics which are open for further investigation.

### 5.6 Concluding Remarks

The field of nickel bioinorganic is entering an exciting period of discovery and understanding. By reading clues from all fields of bioinorganic chemistry, we now possess a deeper knowledge of nickel homeostasis and how the metal is imported, trafficked, stored, regulated, and inserted into nickel-dependent metalloenzymes. However, many details pertaining to the individual
components of these pathways are still missing. In this thesis, the characterization of two metal-binding GTPases, *HpHypB* and *E. coli* YjiA, was presented and the studies demonstrate a unique link between the metal-binding and biochemical properties of these enzymes. The molecular-level details on *HpHypB* presented here afforded the ability to propose a role for this protein in the biosynthetic pathways of urease and [NiFe]-hydrogenase as a means to discriminate metals prior to insertion into the nascent hydrogenase active site. This research will be instrumental in elucidating the maturation pathway of [NiFe]-hydrogenase and urease in *H. pylori* and may help to find a possible target for future therapeutics against this pathogen. In regards to YjiA, although the enzyme lacks a known cellular function, the protein characterization described here will stimulate future research on this largely uncharacterized family of GTPases and focus the directions this research will take. The next steps in understanding these proteins involves a better understanding of the allosteric mechanism, elucidation of the properties of the protein-protein interactions in which these proteins partake and a better understanding of their roles in the cell. Those future projects will find the work here a solid place to begin in those new endeavors.
5.7 References


Appendix I: Elucidation of the Mechanism of Nickel-Induced DNA Binding by *Escherichia coli* NikR: An Attempt to Engineer a Nickel-Independent NikR

**AI.1 Introduction**

Nickel is an essential metal for many bacteria as a critical cofactor of nickel-dependent enzymes. However, the metal is toxic in excess amounts and the cell must closely regulate the intracellular concentrations of nickel. In *Escherichia coli*, the transcription factor NikR is central to this regulation as NikR modulates the expression of the *nikABCDE* operon, encoding a nickel-specific transporter. NikR is a tetrameric protein, consisting of four central metal-binding domains (MBDs) and two flanking ribbon-helix-helix DNA binding domains (DBDs) (Figure AI-1). NikR DNA binding to the *nik* recognition sequence is activated through the binding of 4 nickel ions per tetramer. These nickel ions bind with picomolar affinity at sites located at the center of the protein and are coordinated by His87, His89, and Cys95 from one monomer and His76' from a second monomer, in a square-planar geometry. When nickel binds at this site, termed the “high-affinity” site, NikR can bind the *nik* promoter with a dissociation constant of 5 nM. Additional nickel can also bind to NikR at a second site, with a dissociation constant of 30 nM, tightening the DNA complex to a dissociation constant of 20 pM. The identity of this latter site is not well established, but is believed to consist of His48 and His110 and nearby carboxylate ligands, located between the MBD and DBD.

The mechanism of nickel-activated DNA binding by NikR is not clear. In the crystal structure of metal-free *E. coli* NikR (*Ec*NikR), the two DNA-binding domains are unable to simultaneously bind DNA as they are facing in opposite directions. Upon nickel binding, several elements of the MBD are altered, notably the ordering of the α3-helix, which is disordered in the apo structure. The DNA-bound structure of Ni(II)-NikR illustrates that upon ordering of the α3-helix, two residues are brought into position to form polar, non-specific interactions with the DNA backbone, Lys64 and Arg65. After localization of NikR to the DNA, the protein is believed to undergo a search for the *nik* promoter where the two DBDs form specific contacts.

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5 These experiments were conducted with the support of two undergraduate students under my supervision, Pengpeng Cao and Wasim Kagzi.
with the DNA.\textsuperscript{14} The binding of potassium at a site between the MBDs and DBDs helps to stabilize this conformation and is critical for Ni(II)-responsive DNA binding.\textsuperscript{15,16} Although the crystal structures of NikR support a role for the $\alpha_3$-helix in the mechanism of nickel-activated DNA binding,\textsuperscript{4,9} biochemical evidence is lacking. Previous tryp tic digestion experiments demonstrated that Lys64 and Arg65 in the $\alpha_3$-helix are protected from proteolysis in the presence of Ni(II)\textsuperscript{17} and circular dichroism (CD) experiments reveal a small increase in $\alpha$-helicity upon nickel binding, supporting stabilization of the $\alpha_3$-helix in the presence of nickel.\textsuperscript{6,8} However, these experiments do not prove that stabilization of the $\alpha_3$-helix is the critical

**Figure AI-1. Design rationale for the S69C NikR mutant.** In the structure of apo NikR (top left; PDB: 1Q5V), the two DNA binding domains (DBDs) flanking the core metal-binding domains (MBDs) are free to rotate and the $\alpha_3$-helix is largely unstructured in all but one monomer. Upon the addition of nickel, it is believed that the $\alpha_3$-helices become structured and allow for the protein to localize to DNA by positioning residues to allow for non-specific interactions with the DNA. Two potassium ions also bind to NikR between the DBDs and the MBDs (top right; PDB: 2HZV). In order to stabilize the $\alpha_3$ helix and create a Ni(II)-independent variant of NikR, Ser69 was mutated to a Cys residue thereby allowing a disulfide bond to form between two residues from adjacent monomers, thereby stabilizing the $\alpha_3$ helix (bottom). This bond can be reversibly formed through oxidation and reduction of the disulfide. The color scheme is nickel, green sphere; potassium, purple sphere; sulfur, yellow. The images were generated using PyMol.
component of metal-mediated DNA binding. In order to better understand the role of the α3-helix in Ni(II)-responsive DNA binding by NikR, NikR was modified in an attempt to stabilize the α3-helix and create a NikR capable of Ni(II)-independent DNA binding. A S69C mutant was constructed in order to cross-link the α3-helix from two adjacent monomers via a disulfide bond (Figure A1-1). Although the mutant was successfully expressed and purified, characterization was ambiguous. Based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) assays, an apparent oxidized dimer was obtained. However, the location of the crosslink was not definitely established. Furthermore, this mutant was unable to bind to DNA in the absence of nickel, as monitored by fluorescence anisotropy. These results indicated that the S69C mutation is not sufficient to functionally replace nickel.

AI.2 Materials and Methods

**Materials.** All chemicals were analytical or molecular biology grade and purchased from Sigma-Aldrich unless otherwise noted. All enzymes were from New England Biolabs. Primers were purchased from Sigma Genosys. Ampicillin and iso-propyl-β-D-thiogalactopyranoside (IPTG) were purchased from BioShop (Toronto, Canada). The pNIK103 plasmid used for wild-type NikR was generously donated by Peter Chivers (Washington University School of Medicine, St. Louis, MO). All solutions were prepared with Milli-Q water, 18.2 MΩ-cm resistance (Millipore). For anisotropy experiments, cuvettes were soaked in Sigmacote (Sigma) and rinsed with deionized water prior to use. Electronic absorption measurements were conducted on an Agilent 8453 spectrophotometer with a 1-cm-pathlength cuvette and fluorescence experiments were conducted on a Perkin-Elmer LS-55 Luminescence Spectrophotometer.

**S69C NikR Expression Vector Construction.** The S69C NikR mutant was created from the pNIK103 plasmid by QuikChange PCR mutagenesis (Statagene) with *Pfu* Turbo polymerase by using the forward and reverse primers, 5′GAAAAACGCGACTTAGCCTGCCGATTGTCTC$^\text{CAC}^3′$ and 5′GTGGAGACAATGCAGCCAGCTAAGTTCGCGTTTTTT$^3′$, respectively. The template strand was subsequently digested with *DpnI*. For production of large amounts of the parent pNIK103 and mutant plasmid, the 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, Toronto, Canada) in the forward and reverse directions by using the T7 promoter and terminator primers.

**Protein Expression and Purification.** For expression of wild-type (WT) and mutant NikR, the plasmids were transformed into BL21 Star (DE3) *E. coli* cells (Invitrogen). Overnight cultures were grown, and 25 mL was used to inoculate 1.5 L of LB medium supplemented with 100 μg/mL ampicillin. The cells were grown aerobically at 37 °C until the A600 reached 0.6, at which point they were induced with 0.3 mM IPTG. After shaking at 37 °C for an additional 3 h for WT or at 25 °C for an additional 5 h for S69C, the cells were harvested by centrifugation and resuspended in 30 mL 20 mM Tris(hydroxymethyl)aminomethane (Tris), pH 7.5, and 100 mM NaCl. For a single purification of WT NikR, a total of 3 L of cell culture was used, whereas a total of 6 L of cell culture was used for S69C. All subsequent steps were performed at 4 °C or on ice. The resuspended cells were sonicated and centrifuged at 25,000 × g for 40 min. NikR was purified on a Ni-NTA column (Qiagen) as previously described. Following the Ni-NTA column, the eluted protein was dialyzed against 20 mM Tris, pH 7.6, and 5 mM EDTA overnight. Purifications of WT NikR also included 1 mM DTT in the dialysis buffer. The protein solution was loaded onto a MonoQ HR 10/10 FPLC column (GE Healthcare) in 20 mM Tris, pH 7.5, and eluted with a linear NaCl gradient (NikR eluted near 350 mM NaCl). The fractions were analyzed using 12.5 % SDS-PAGE and pure protein was pooled. The molecular mass of WT and mutant NikR were confirmed by electrospray ionization mass spectrometry (ESI-MS; Department of Chemistry, University of Toronto). The determined molecular masses of WT and S69C NikR were 15094.0 and 15110.0 Da, which correspond to their calculated molecular masses of 15093.7 and 15109.8 Da, respectively. The protein concentrations were calculated by using the extinction coefficient of 4470 M⁻¹ cm⁻¹ for both WT and S69C NikR at 280 nm in Milli-Q water. All proteins were > 90 % pure as estimated by Coomassie-stained SDS-PAGE that was analyzed by using the public domain NIH ImageJ program (developed at the U.S. National Institutes of Health and available on the Internet at [http://rsb.info.nih.gov/ij/](http://rsb.info.nih.gov/ij/)). The absence of any bound metal to the protein was confirmed by a 4-(2-pyridylazo)-resorcinol (PAR) assay, in which the protein was denatured with 4 M guanidinium hydrochloride (GuHCl) and 50 μM PAR was added to the sample. The absorbance at 500 nm, corresponding to the formation of a 2:1 PAR-Me(II) complex, was monitored and compared to a standard curve prepared with 50 μM PAR in 4 M GuHCl and known metal concentrations. The free thiol content of the
proteins was quantified via reaction of the protein with DTNB in the presence of 6 M GuHCl 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 Oxidation Methods.** In order to obtain the desired disulfide bond in the engineered S69C mutant, several different methods of protein oxidation were attempted. In all cases, the effectiveness of disulfide bond formation was monitored by screening the protein on a 12.5% SDS-PAGE gel, in the absence of reducing agent, and with a DTNB assay.

**Cu(II) Oxidation.** Based on the method of Ahmed et al.,20 75-100 µM apo-S69C NikR was incubated with 50-75 µM CuSO₄ in protein buffer exposed to air at room temperature. After 1 h, 50 mM EDTA was added and the sample was incubated overnight at 4 °C. The Cu(II) and EDTA were removed by passing the sample through a PD-10 gel filtration column (GE Healthcare) equilibrated with protein buffer (20 mM Tris, pH 7.5, 100 mM KCl).

**GSH/GSSG Oxidation.** The oxidation of S69C NikR through the use of reduced and oxidized glutathione was based on previous work by Ahmed et al.20 and Wetlaufer et al.21 Samples of 100 µM holo-S69C NikR in protein buffer were incubated with 40 mM GSH and 8 mM GSSG, such that the ratio of [GSH]/[GSSG] = 5 and that the [GSH] = 400 × [NikR] and [GSSG] = 80 × [NikR], as recommended by Wetlaufer et al.21 The sample was incubated for 1-3 hours at room temperature, after which the GSH and GSSG were removed either by PD-10 or by loading the protein onto a MonoQ column as described above.

**Hydrogen Peroxide Oxidation.** Samples of 100 µM S69C NikR were incubated with 0.2-1 mM H₂O₂ in protein buffer for 1.5-3 h at room temperature. The protein was then passed through a PD-10 column to remove the hydrogen peroxide.

**DTNB Oxidation.** Villafranca et al. described a method to oxidize an engineered disulfide bond using DTNB.22 Samples of 100 µM holo-S69C NikR were incubated with 20-5000 µM DTNB at either room temperature or 37 °C for 2 h. Some samples also contained 90-2000 µM DTT. The DTT and DTNB were removed by passing the protein through a PD-10 column.

**Nickel Binding Titrations.** A sample of 10-25 µM apo WT or S69C NikR was prepared in protein buffer supplemented with 10 mM glycine, to which increasing amounts of NiCl₂ were
added. After addition of nickel, the sample was allowed to equilibrate at room temperature for 15 minutes prior to monitoring the electronic absorption spectrum between 250-500 nm. The samples were corrected by background subtraction at 600 nm.

**Circular Dichroism (CD) Spectroscopy.** WT and S69C NikR samples were prepared for CD spectroscopy by diluting the protein in MilliQ water to a final concentration of approximately 10-20 µM in the glovebox. For samples containing nickel, an equimolar amount of NiCl₂ was added to the diluted samples and allowed to equilibrate overnight at 4°C in the glovebox. All samples were analyzed on an Olis RSM 1000 spectropolarimeter with a capped 1 mm pathlength cuvette in order 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 seconds and 2400 grating lines per nm. The final spectra obtained are averages of three scans. The observed ellipticity was converted into mean residue ellipticity ([θ]ₘᵣₑ; deg cm² dmol⁻¹) using the following formula:

\[
[\theta]_{mre} = \frac{(MW/N-1) \times \theta}{[protein] \times l \times 10}
\]

where MW is the molecular weight of the protein in Da, N is the number of amino acids, θ is the observed ellipticity in degrees, [protein] is the concentration of protein in g/mL and l is the pathlength.

**Fluorescent 54 bp DNA Probe Preparation.** The fluorescent NikR operator DNA probe (54mer) containing the nik recognition sequence was purchased as two single strands from IDT Technologies. The sequences of the two strands are 5'GAACAGGTAATCAGTAGACGAAT ACT/iFluorT/AAAATCGTCATACTTATTTCCGCCAT3' and 5'ATGGCCGAAATAAGTATG ACGATTTTAAAGTATTCGCATACTGATTACCTGTTC3', where iFluorT is a fluorescein label conjugated to a thymine base. Double-stranded 54mer was made by annealing 100 µM of each strand dissolved in annealing buffer (10 mM Tris, pH 7.6, 50 mM NaCl) by heating the mixture at 95 °C for 10 min and then allowing it to cool to room temperature overnight. Single stranded DNA was removed by HPLC on a reverse-phase C18 Zorbax 300-SB column (Agilent). An aliquot of the annealed 54 bp DNA was loaded onto the column equilibrated with 50 mM triethylammonium acetate, pH 7.0, and eluted by using a linear gradient of 100 % acetonitrile
(double stranded DNA eluted at approximately 13 % acetonitrile). The absorbance at 260 nm and 490 nm (for the fluorescein labeled DNA) was monitored over the course of the gradient.

To check for probe purity, an aliquot of 54mer was radiolabelled with \([\gamma-^{32P}]ATP\) by T4 polynucleotide kinase by incubating together in 1 X PNK buffer (New England Biolabs) for 2 h at 37 °C. Unincorporated nucleotides and the kinase were removed by centrifugation with a G-25 microspin column (GE Healthcare). The radiolabelled product was run on a 7 % native polyacrylamide gel and exposed to a phosphor screen for 20 minutes. The screen was scanned on an Amersham Biosciences Storm 860 phosphorimager and the gel image was analyzed using ImageJ. The probe was quantitated by measuring the absorbance at 260 and 494 nm of the probe in protein buffer at pH 7.6 and by using the extinction coefficients \(\epsilon_{260} = 1,074,200 \text{ M}^{-1} \text{ cm}^{-1}\) and \(\epsilon_{494} = 68,000 \text{ M}^{-1} \text{ cm}^{-1}\).

**Fluorescence Anisotropy.** For all anisotropy experiments, the fluorescence cuvette was pretreated with Sigmacote to prevent adherence of protein or DNA to the walls. The 54mer was diluted to 5 nM in binding buffer (20 mM Tris, pH 7.5, 100 mM KCl, 3 mM MgCl\(_2\), 0.1 % IGEPAL, 5 % glycerol, 0.1 mg/mL bovine serum albumin, and 0.1 mg/mL sonicated herring-sperm DNA). Increasing amounts of apo- or holo-WT or S69C NikR were added to the 54mer and the fluorescence anisotropy at 515 nm was monitored upon excitation at 495 nm with an integration time of 3 seconds. Each anisotropy measurement was made 4 times for each protein concentration. After each addition of protein, the solution was allowed to equilibrate at room temperature for 10 minutes. The raw anisotropy values were converted into fraction DNA bound, \(r\), by using the following formula:

\[
r = \frac{I_{\text{obs}} - I_{\text{min}}}{I_{\text{max}} - I_{\text{min}}}
\]

where \(I_{\text{obs}}\) is the observed anisotropy value at a given protein concentration; \(I_{\text{min}}\) is the anisotropy of the free 54mer, and \(I_{\text{max}}\) is the anisotropy upon saturation. The free protein concentration was determined by subtracting the amount of DNA-bound protein from the total amount of protein added. The DNA-bound fraction was plotted as a function of free protein concentration and the data were fit using OriginPro8 to the Hill coefficient:
\[ T = \frac{[\text{NikR}]^{n}_{\text{free}}}{K_d^{n} + [\text{NikR}]^{n}_{\text{free}}} \]

where \( n \) is the Hill coefficient.

**GluC Digestion of NikR and MALDI-MS.** Samples of 30-120 µM NikR were incubated with 10 µM GluC (New England Biolabs) in 0.5 X GluC buffer at room temperature for 2-18 h. Peptide-containing solution (1 µL) was mixed with 1 µL of 4-hydroxy-R-cyanocinnamic acid matrix solution (Thermo Scientific; prepared according to manufacturer’s instructions) and 1 µL 0.1 % trifluoroacetic acid directly on the MALDI plate. MALDI-time-of-flight mass spectrometry (MALDI-TOF-MS) analyses were performed using a Waters Micromass MALDI micro MX mass time-of-flight spectrometer equipped with a UV-nitrogen laser (337 nm) and a microchannel plate detector. The molecular weight determination acceleration voltage was set to 25 kV and the instrument was run in reflectron mode.

**Al.3 Results**

**Design Rational of the S69C NikR Mutant.** The previously determined crystal structure of NikR in three different states, in the absence of metal, in complex with stoichiometric nickel, and in a complex with stoichiometric nickel and the DNA recognition sequence,\(^4\,9\) revealed a possible mechanism of nickel-induced DNA-binding. The crystal structure revealed that in the absence of metal, the α3-helix and preceding loop region in the metal-binding domain (MBD) are unstructured, but upon nickel-binding to the high-affinity nickel site, the α3-helix and loop region are stabilized.\(^4\) The stabilization of this region may contribute to DNA binding through two major mechanisms, notably through the positioning of Lys64 and Arg65, two polar residues critical for interacting with the phosphate backbone of the DNA\(^13\) and stabilization of a network of residues which stabilize features in the MBD and in turn modulate DNA binding. To test this hypothesis, engineering of NikR was attempted in order to stabilize the α3-helix in the absence of nickel.

The structure of the NikR-DNA complex reveals that across the interface between monomers in NikR, the side-chain oxygens of the Ser69 residues on the α3-helix are less than 4 Å apart and upon rotation around the Co-Cβ bond, they come within 2 Å. Thus, upon mutating these Ser
residues to Cys residues, a disulfide bond may form and lock the α3-helices in the optimal DNA-binding conformation, even in the absence of nickel (Figure A1-1). Using a disulfide to stabilize the α3-helix has the benefits of being genetically incorporated into the protein and allows for the addition of reducing agent to reduce the disulfide thereby allowing the α-helix to unfold in the absence of nickel, and in so doing, disfavoring DNA binding. This system would allow for the nickel-independent control over the DNA-binding properties of NikR. NikR possesses two native cysteines, of which the closest to Ser69 is the nickel ligand Cys95. However, this residue is 13 Å away based on distance between α-carbons, so unwanted disulfides should not form if the protein is properly folded.

**Disulfide Bond Oxidation.** Upon screening purified S69C NikR on a non-reducing SDS gel, two bands were visible, corresponding to the monomeric and dimeric species (Figure A-2). The gel, run under denaturating conditions, should have broken any non-covalent complexes, thus

![Figure A1-2. Oxidation of S69C NikR using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB).](image)

Purified S69C NikR (lane 2) was found to be approximately 50% oxidized based on SDS-PAGE and DTNB assay analysis. In an attempt to increase the amount of cross-linked dimer, several oxidation strategies were utilized, the most promising of which was oxidation with DTNB (see text for details). Two temperatures were attempted, room temperature and 37 °C, with an incubation period of 30 minutes. At each temperature, the ratio of DTNB:thiolate was varied between 0.25 – 10 in order to ascertain the ideal ratio (black bars). The most promising results were obtained at room temperature with a DTNB:thiolate of 1:3. The first lane was loaded with molecular weight markers, with the corresponding molecular weights listed at the left. The gel was a 12.5% denaturing gel. Monomeric S69C NikR migrates slightly faster than its MW (15.1 kDa) and is the lowest band on the gel. Dimeric S69C NikR migrates at approximately 25.0 kDa. The gel was cropped, brightened and contrast adjusted for clarity.
indicating that the detected dimeric species is likely that of cross-linked S69C NikR. However, a significant portion of the protein was found to be un-oxidized (Figure AI-2), suggesting that exposure to air was insufficient to yield a large amount of cross-linked S69C NikR. In an attempt to yield a larger portion of oxidized mutant, several oxidation methods were attempted: Cu(II)-catalyzed oxidation, incubation with GSH/GSSG, hydrogen peroxide, and DTNB reduction.

The Cu(II) oxidation yielded a larger proportion of dimer compared to monomer, but the method was ultimately abandoned due to the inability to remove all of the bound copper from NikR, as Cys \( \rightarrow \) Cu(II) LMCT bands were evident in the electronic absorption spectrum of the S69C mutant after treatment with EDTA (data not shown). The GSH/GSSG oxidation method did not yield increased dimer formation. Furthermore, ESI-MS and MALDI-MS revealed several protein-GSH adducts (Table AI-1). GluC digestion and subsequent MALDI-MS of the

<table>
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<th>Oxidation Method</th>
<th>Observed Mass (Da)</th>
<th>Calculated (Da)</th>
<th>Assignment</th>
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</thead>
<tbody>
<tr>
<td>GSH/GSSG</td>
<td>15110.0</td>
<td>15109.8</td>
<td>S69C Monomer</td>
</tr>
<tr>
<td></td>
<td>15416.0</td>
<td>15415.1</td>
<td>S69C Monomer + GSH</td>
</tr>
<tr>
<td></td>
<td>30218.0</td>
<td>30217.6</td>
<td>S69C Dimer</td>
</tr>
<tr>
<td></td>
<td>30522.0</td>
<td>30522.9</td>
<td>S69C Dimer + GSH</td>
</tr>
<tr>
<td></td>
<td>30830.0</td>
<td>30828.2</td>
<td>S69C Dimer + 2 GSH</td>
</tr>
<tr>
<td>DTNB and DTT</td>
<td>15109.0</td>
<td>15109.8</td>
<td>S69C Monomer</td>
</tr>
<tr>
<td></td>
<td>15306.0</td>
<td>15307.0</td>
<td>S69C Monomer + NTB</td>
</tr>
<tr>
<td></td>
<td>30218.0</td>
<td>30217.6</td>
<td>S69C Dimer</td>
</tr>
<tr>
<td></td>
<td>30414.0</td>
<td>30414.8</td>
<td>S69C Dimer + NTB</td>
</tr>
<tr>
<td></td>
<td>30612.0</td>
<td>30612.0</td>
<td>S69C Dimer + 2 NTB</td>
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</table>

†The molecular masses of monomeric and dimeric S69C NikR are 15109.8 and 30217.6 Da, respectively. The molecular mass of the glutathione adduct is 307.3 Da and the molecular mass of the 2-nitro-5-thiobenzoic acid adduct (NTB) is 199.2 Da.
GSH/GSSG oxidized protein yielded a $m/z$ peak at 2489.8, corresponding to the peptide fragment Lys64-Asp80 + GSH ($m/z_{\text{calc}} = 2487.7$), suggesting that Cys69 is one of the residues forming an adduct with glutathione. Due to the lack of oxidation and the formation of adducts, this method was also abandoned. The hydrogen peroxide method failed to yield any increase in the amount of dimer.

Initial tests demonstrated that the DTNB method was the most efficient at producing dimeric S69C NikR. When incubated over a 3 day period at room temperature, > 85% of the protein was oxidized to dimeric protein (Figure AI-2). When a low ratio of DTNB:thiolate was used (< 3 DTNB:thiolate), a larger species, roughly corresponding to a tetramer was also noted in the gel, but as the DTNB:thiolate ratio increased, this larger species was no longer observed (Figure A-2). Despite the promise of this oxidation method, it was ultimately abandoned due to protein adducts as determined by ESI-MS (Table AI-1). The increase in the mass suggested that these adducts are due to covalent attachment of 2-nitro-5-thiobenzoic acid to the Cys residues.

**S69C NikR behaves similarly to WT NikR.** Despite the lack of fully oxidized S69C NikR, we proceeded with experimentation with S69C NikR on the rational that although not all the protein was oxidized, it was clear from the SDS-PAGE and MS results that a significant portion of the protein was an oxidized dimer even without treatment and this mixture should yield some information.

Upon the addition of Ni(II) to oxidized S69C NikR, an increase in the 302 nm signal was observed, similar to what is observed for WT NikR (Figure AI-3 inset). This band has been previously assigned to a Cys$\rightarrow$Ni(II) LMCT$^{6,8}$ and was linear up to 1 equivalent of metal, yielding an extinction coefficient of $6900 \pm 800 \, \text{M}^{-1} \, \text{cm}^{-1}$ (Figure AI-3). This is in comparison to the previously reported extinction coefficient of $7200 \, \text{M}^{-1} \, \text{cm}^{-1}$ for WT NikR,$^8$ suggesting that the Ni(II)-coordination sphere is not significantly perturbed by the mutation or disulfide cross-linking.

Circular dichroism was utilized to detect changes in the alpha helical content of the protein. In the presence of nickel, an increase in overall alpha helical content was observed for WT NikR (Figure AI-4) as previously reported.$^6$ If our engineered disulfide stabilizes the $\alpha3$-helix, a similar increase in alpha helical content should be observed. The CD spectrum of the reduced S69C NikR mutant is almost identical to that of apo-WT NikR (Figure AI-4), suggesting similar
amoun...208 and 222 nm, indicative of increased α-helix content. In contrast, oxidized S69C NikR features an increase in the signal at 208 and 222 nm, indicative of increased α-helix content.23,24

In order to establish the position of the disulfide within the S69C mutant, protease digestion of NikR, followed by MALDI mass spectrometry, was utilized in an attempt to isolate the disulfide-linked peptide fragments of NikR. The endopeptidase GluC was selected as it was previously shown to produce reasonably sized NikR peptide fragments which should be readily detectable in the MS.17 However, we were unable to detect the disulfide-linked peptide in the MALDI spectra (data not shown).

**S69C NikR is unable to bind to the nik promoter in the absence of nickel.** Fluorescence anisotropy was used to probe the interaction between reduced- and oxidized-S69C NikR and the nik promoter sequence in the absence of nickel. In the absence of nickel, NikR does not bind to the DNA probe and no anisotropy change was observed (data not shown). When stoichiometric

Figure AI-3. Nickel titration and difference spectrum of S69C NikR. The difference spectra (grey line; inset), generated by subtracting the spectrum of 10 µM S69C NikR loaded with NiCl₂ from that of apo-S69C, yields an intense band centered at 302 nm, assigned to a Cys → Ni(II) LMCT. This band is similar to that observed with WT NikR, prepared under the same conditions (black line; inset). Ni(II) binding to 20 µM S69C NikR can be monitored at 302 nm (circles) and the resultant plot is linear up to 1 equivalent (fit, dotted line), yielding an extinction coefficient of 6900 ± 800 M⁻¹ cm⁻¹, similar to that for WT NikR, 7200 M⁻¹ cm⁻¹, as previously published.8
nickel was added to the protein, WT NikR binds to the DNA, and a change in anisotropy was observed. The anisotropy change was converted into a fractional saturation of the DNA probe, allowing the data to be fit to an apparent $K_d = 39 \pm 10 \text{ nM}$, $n = 1.07 \pm 0.04$ (Figure A-5), similar to previously reported values. The holo-S69C mutant, in the oxidized and reduced forms, binds with affinities similar to that of holo-WT, with an apparent $K_d = 31 \pm 3 \text{ nM}$ ($n = 1.0 \pm 0.2$) and $38 \pm 3 \text{ nM}$ ($n = 1.1 \pm 0.1$), respectively (Figure AI-5). This suggests that despite the engineered disulfide bond, the protein’s DNA-binding ability is not perturbed. In the absence of nickel, neither the reduced nor oxidized S69C NikR could bind to the DNA probe and no anisotropy change was observed, suggesting that the engineered mutant still requires nickel for DNA binding.

Figure AI-4. Circular dichroism (CD) spectra of WT and S69C NikR. The CD spectrum of apo-WT NikR (red circles) displays an overall shape indicative of a mixed α/β protein. Upon the addition of Ni(II) (blue triangles), the intensity of the spectrum increases, notably at 208 and 222 nm, suggesting an increase in α-helical content. Apo, reduced S69C NikR (black circles), is similar in overall secondary structure to that of WT NikR. Addition of nickel to the reduced protein (black squares) features an increase in the 208 and 222 nm features, signaling an increase in α-helix content. A similar increase is noted in oxidized S69C NikR, even in the absence of metal (green squares).
The role of the \( \alpha_3 \)-helix in Ni(II)-responsive DNA binding by NikR has been suggested by multiple experiments, including crystallography,\(^4,9\) tryptic digestion studies,\(^17\) and observed changes in the circular dichroism spectrum of NikR,\(^6,8\) but no study has yet investigated the role of this helix directly. Here, we describe an attempt to create a Ni(II)-independent variant of NikR which utilizes an engineered disulfide bond to stabilize the \( \alpha_3 \)-helix. Several other studies have utilized disulfides as a means to cross-link structural components of proteins.\(^{25-27}\) It is interesting that in none of these studies did the authors need to resort to extra steps to oxidize their engineered disulfide bonds. For example, in the case of Al-Rabiee et al., the authors utilized a disulfide bond to successfully reproduce catalytic inhibition in the absence of inhibitor in the enzyme D-3-phosphoglycerate dehydrogenase and they found exposure to air at 4 °C was sufficient to obtain the appropriate disulfide bond.\(^{25}\) The poor disulfide-bond formation of our S69C mutant suggests that other factors are at play. It is conceivable that the Cys69 residues in

**Figure AI-5. Binding of nickel-loaded WT and S69C NikR to the nik promoter.** Fluorescence anisotropy was used to monitor the binding of WT and S69C NikR, loaded with stoichiometric metal, to a 54 bp DNA fragment containing the \( nik \) recognition sequence. Holo WT NikR (circles; fit, solid line) binds with an apparent \( K_d = 39 \pm 10 \text{ nM} \) \((n = 1.07 \pm 0.04)\). The affinities of holo-oxidized (squares; fit, dashed line) and holo-reduced (triangles; fit, dotted line) S69C were determined to be \( K_d = 31 \pm 3 \text{ nM} \) \((n = 1.0 \pm 0.2)\) and \( 38 \pm 3 \text{ nM} \) \((n = 1.1 \pm 0.1)\), respectively. Apo oxidized- and reduced-S69C NikR did not yield an anisotropy change. Representative data sets are shown.

### AI.4 Discussion

The role of the \( \alpha_3 \)-helix in Ni(II)-responsive DNA binding by NikR has been suggested by multiple experiments, including crystallography,\(^4,9\) tryptic digestion studies,\(^17\) and observed changes in the circular dichroism spectrum of NikR,\(^6,8\) but no study has yet investigated the role of this helix directly. Here, we describe an attempt to create a Ni(II)-independent variant of NikR which utilizes an engineered disulfide bond to stabilize the \( \alpha_3 \)-helix. Several other studies have utilized disulfides as a means to cross-link structural components of proteins.\(^{25-27}\) It is interesting that in none of these studies did the authors need to resort to extra steps to oxidize their engineered disulfide bonds. For example, in the case of Al-Rabiee et al., the authors utilized a disulfide bond to successfully reproduce catalytic inhibition in the absence of inhibitor in the enzyme D-3-phosphoglycerate dehydrogenase and they found exposure to air at 4 °C was sufficient to obtain the appropriate disulfide bond.\(^{25}\) The poor disulfide-bond formation of our S69C mutant suggests that other factors are at play. It is conceivable that the Cys69 residues in
solution are not as close together as was suggested in the crystal structure, thereby making disulfide bond formation more difficult, even in the Ni(II)-bound state. For example, computational analysis of *H. pylori* NikR (*Hp*NikR) demonstrated that even in the presence of Ni(II), the protein is sampling many different conformational states. Likewise, *Ec*NikR in solution is likely to feature a fluid structure, switching between many different conformations. Although these changes in conformational state are likely to be small and not significantly affect the overall structure of NikR, as was observed in *Hp*NikR, they may be sufficient to cause issues when attempting to obtain a significant population of cross-linked NikR.

The lack of DNA binding by apo, oxidized-S69C NikR suggests that either the formation of the α3-helix is not critical to DNA binding or that the engineered disulfide bond was insufficient to ensure stabilization of this secondary structure element. Based on the circular dichroism spectrum, an increase in α-helical content was achieved. However, it is unclear if this is due to stabilization of the α3-helix in particular. Furthermore, it is possible that ordering of the α3-helix is not sufficient to activate DNA binding. For example, ordering of the α3-helix was also noted in the structure of the MBD loaded with Cu(II), but Cu(II)-bound NikR is unable to bind to DNA with the same affinity at the Ni(II)-loaded protein, suggesting that there are additional interactions within the protein induced by Ni(II)-binding that extend beyond those of the α3-helix. Without nickel, these interactions would be absent, so even with a cross-linker present, this region of the protein would be unable to obtain the α-helical secondary structure.

The data presented here demonstrate that this engineered S69C NikR variant is incapable of Ni(II)-independent DNA binding. However, the reasons for this lack of function are unclear and a clear link between stabilization of the α3-helix upon Ni(II) binding and DNA binding cannot be established. Ongoing research is trying alternate avenues to stabilize the α3-helix. These avenues include methods such as the incorporation of photo-responsive cross-linkers and the replacement of residues in the α3 helix with residues with high α-helix propensities in order to stabilize the helical structure. The research presented here should serve as a reference for design considerations and assist in the interpretation of future experiments geared towards elucidating the role of the α3-helix in NikR’s Ni(II)-induced DNA binding.
AI.5 References


Refolding Rate of a Small Protein Controlled by Engineered Disulfide Bonds, *Biophys. J.* 92, 225-233.


Appendix II: DYNAFIT Scripts

AII.1 Competition Between $HpHypB$ and MF2 for Ni(II)

[task]
  data = equilibria
  task = fit

[mechanism]
  M + MF2 $\rightleftharpoons$ M.MF2 : Kd1  dissoc.
  M + HpHypB $\rightleftharpoons$ M.HpHypB : Kd2  dissoc.

[concentrations]
  MF2 = 10
  HpHypB = 10

[constants]
  Kd1 = .15
  Kd2 = .036 ?

[responses]
  MF2 = 0.11172 ?
  M.MF2 = 0.29539 ?

[equilibria]
  variable M
  offset auto ?
  file ./documents/HpHypB/ dat.txt

[output]
  directory ./output/HpHypB/

[end]
All.2  \textit{HpHypB Ni(II) vs. Zn(II) Competition}

\begin{verbatim}
[task]
data = equilibria
  task = fit

[mechanism]
  Ni + HpHypB <=> Ni.HpHypB : Kd1 dissoc.
  Zn + HpHypB <=> Zn.HpHypB : Kd2 dissoc.

[concentrations]
  HpHypB = 10
  Ni = 50

[constants]
  Kd1 = .15
  Kd2 = .036 ?

[responses]
  Ni.HpHypB = 5.80E-02 ?
  Zn.HpHypB = 5.91E-03 ?

[equilibria]
  variable Zn
  offset auto ?
  file ./documents/HpHypB/Zinc/dat.txt

[output]
  directory ./output/HpHypB/Zinc/

[end]
\end{verbatim}
All.3  Competition Between Mg(II)-loaded \( HpHypB \) and MF2 for Ni(II)

(task)
\begin{verbatim}
data = equilibria
\end{verbatim}
(task = fit)

(mechanism)
\begin{verbatim}
M + MF2 <==> M.MF2 : Kd1  dissoc.
M + HpHypB <==> M.HpHypB : Kd2  dissoc.
\end{verbatim}

(concentrations)
\begin{verbatim}
MF2 = 5
HpHypB = 10
\end{verbatim}

(constants)
\begin{verbatim}
Kd1 = 0.7
Kd2 = 1 ?
\end{verbatim}

(responses)
\begin{verbatim}
M.MF2 = -104780 ?
\end{verbatim}

(equililibria)
\begin{verbatim}
variable M
\end{verbatim}
\begin{verbatim}
offset auto ?
\end{verbatim}
\begin{verbatim}
file ./DynaFitDocs/HpHypB/MgKCl/dat.txt
\end{verbatim}

(output)
\begin{verbatim}
directory ./DynaFitOutput/HpHypB/MgKCl/
\end{verbatim}

[end]
All.4  Competition Between Nucleotide-loaded HpHypB and MF2 for Ni(II)

[task]
   data = equilibria
   task = fit

[mechanism]
   M + MF2 ⇌ M.MF2 : Kd1  dissoc.
   M + HpHypB + HpHypB ⇌ M.HpHypB.HpHypB : Kd2  dissoc.

[concentrations]
   MF2 = 5
   HpHypB = 10

[constants]
   Kd1 = 1.849 ;Kd1 = 1.8 and 1.5 for samples containing GDP and GDPNP, respectively
   Kd2 = 1 ?

[responses]
   M.MF2 = -104780 ?

[equilibria]
   variable M
   offset auto ?
   file ./DynaFitDocs/HpHypB/GdpNi/dat.txt

[output]
   directory ./DynaFitOutput/HpHypB/GdpNi/

[end]
AII.5 Competition Between Mg(II)- and Nucleotide-loaded HpHypB and MF2 for Zn(II)

[task]

data = equilibria
task = fit

[mechanism]

\[ Zn + MF2 \iff ZnMF2 : Kd1 \text{ dissoc.} \]
\[ Zn + HpHypB \iff Zn.HpHypB : Kd2 \text{ dissoc.} \]

[concentrations]

HpHypB = 5
MF2 = 5

[constants]

Kd1 = .27 ; Kd1 = 0.27, 0.59, and 0.38 in the presence of Mg(II), GDPNP, and GDP, respectively
Kd2 = .036 ?

[responses]

ZnMF2 = 2167682 ?

[equilibria]

variable Zn
offset auto ?
file ./DynaFitDocs/HpHypB/KClZnGDP/dat.txt

[output]

directory ./DynaFitOutput/HpHypB/KClZnGDP/

[end]