Probing the link between Ni(II) binding and GTP hydrolysis by the

*Escherichia coli* metal chaperone HypB

by

Conor Jack Zeer-Wanklyn

A thesis submitted in conformity with the requirements
for the degree of Master of Science

Graduate Department of Chemistry
University of Toronto

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University of Toronto
2017

**Abstract**
Nickel enzymes are virulence factors in some human pathogens. Oftentimes the maturation machinery that forms these enzymes includes a nickel chaperone that is also an NTPase. In [NiFe]-hydrogenase maturation, this NTPase factor is HypB, which hydrolyzes GTP, binds nickel, and in the GDP state delivers nickel to the partner protein HypA. One metal-binding residue, C198 in *Escherichia coli*, is in the Switch II GTPase motif, and possibly connects the metal-binding, nucleotide-binding, and GTP hydrolyzing activities of HypB. A C198T mutation, made in an effort to disconnect these processes, prevented HypB from maturing [NiFe]-hydrogenase in *E. coli*. C198T HypB could bind nickel with low μM $K_d$, hydrolyze GTP, and GTP hydrolysis remained unexpectedly metal sensitive. The C198T mutation may have affected nucleotide-dependent metal affinity, or GDP-dependent complex formation with HypA; however, these possibilities need to be further explored. This work contributes to our understanding of the role of the NTPase in metallocenter assembly.
Acknowledgements

I am glad to have this space to express my gratitude to Professor Deborah Zamble - thank you for your mentorship, and for making me into a scientist. Thank you for teaching me how to conduct and communicate research, and for giving me the space to learn from mistakes. I would also like to thank Professor Jumi Shin, my second reader, for her advice on how to improve this document.

I am grateful for my lab mates, for their insight and help, and for all the fun we’ve had together! In particular I would like to thank Michael Lacasse for showing me the ropes as an undergraduate student in the lab.

I would like to thank my wonderful brothers, Issac Zeer-Wanklyn, Lucas Zeer-Wanklyn, Brayden Zeer, and Mitchel Zeer. I appreciate your comaraderie and care, and for being people with whom I can share so openheartedly. You’ve been such great pals, I love you all, and I am proud of you all!

I would also like to thank my mom, Jill Wanklyn, and dad, Rob Zeer, for their inspiration, council, and fervent support throughout the last few decades. Thank you for believing in me, surrounding me with love, and providing me with the tools to succeed. I am also so grateful for my step-mom Corina Hierl. I treasure our time together, and am touched by your unhesitating affection and support- ich liebe dich!

No one does it alone, and I’m fortunate to have you all at my side. Thank you!
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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethylaminoethyl</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5-dithiobis(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>electrospray ionization-mass spectrometry</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>GTP&lt;sub&gt;a&lt;/sub&gt;</td>
<td>GTP analogue</td>
</tr>
<tr>
<td>Gpp(NH)p</td>
<td>guanosine 5'-[β,γ-imido] triphosphate</td>
</tr>
<tr>
<td>GuHCl</td>
<td>guanidinium hydrochloride</td>
</tr>
<tr>
<td><em>H. pylori</em></td>
<td><em>Helicobacter pylori</em></td>
</tr>
<tr>
<td>Hepes</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HypA</td>
<td>hydrogenase pleotropic A</td>
</tr>
<tr>
<td>HypA&lt;sub&gt;str&lt;/sub&gt;</td>
<td>Strep-tag II-recombinant HypA</td>
</tr>
<tr>
<td>HypB</td>
<td>hydrogenase pleotropic B</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>K\textsubscript{D}</td>
<td>apparent dissociation constant</td>
</tr>
<tr>
<td>KPB</td>
<td>potassium phosphate buffer</td>
</tr>
<tr>
<td>LB</td>
<td>lysogeny broth</td>
</tr>
<tr>
<td>LMCT</td>
<td>ligand-to-metal charge-transfer</td>
</tr>
<tr>
<td>MF2</td>
<td>mag fura 2</td>
</tr>
<tr>
<td>NTP</td>
<td>nucleoside triphosphate</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAR</td>
<td>4-(2-pyridylazo)-resorcinol</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>P\textsubscript{i}</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>PMB</td>
<td>(p)-hydroxymercuribenzoic acid</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SlyD</td>
<td>sensitive to lysis D</td>
</tr>
<tr>
<td>TCEP</td>
<td>tris(2-carboxyethyl)phosphine</td>
</tr>
<tr>
<td>T\textsubscript{m}</td>
<td>melting temperature</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TYEP</td>
<td>tryptone yeast-extract phosphate</td>
</tr>
<tr>
<td>TYET</td>
<td>tryptone yeast-extract Tris</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>ultraviolet-visible</td>
</tr>
</tbody>
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Introduction†

Nickel is a micronutrient used by a wide variety of organisms, which incorporate the metal ion into the catalytic centers of enzymes [1,2]. To date, nine nickel enzymes have been identified, and these systems play key roles in global nitrogen, carbon, and hydrogen cycles [1,3,4]. These systems are also implicated as virulence factors in human pathogens, including *Helicobacter pylori* and *Escherichia coli* [5-7]. Both *H. pylori* and *Enterobacteriaceae* were recently named by the World Health Organization as priority pathogens for antibiotic research and development [8]. In the case of *H. pylori*, two nickel enzymes, urease and [NiFe]-hydrogenase, are required for colonization of the mammalian stomach [5].

Although nickel allows microbes to access chemistry that is important for survival and colonization, the transition metal can also have toxic effects. Nickel ions can interact with non-metalloenzymes, affecting their activity, or replace essential metals in metalloenzymes, thereby inactivating them [9]. For instance, in *E. coli*, nickel toxicity arises in part because nickel specifically replaces one of the zinc ions in fructose-1,6-bisphosphate aldolase. This substitution causes loss of an enzymatic activity required for glycolysis [10].

Organisms are able balance this dual nature of nickel, nutrient and toxin, by the use of multiple auxiliary pathways [5,11]. These systems include transporters that control

† Figures and text have been adapted from Current Opinion in Chemical Biology, 37, Microbial nickel: cellular uptake and delivery to enzyme centers, Zeer-Wanklyn, C. J., Zamble, D. B., 80-88, 2017 with permission from Elsevier.
uptake and efflux of nickel ions across cell membranes, intracellular nickel-binding proteins involved in metal ion distribution and storage, as well as regulatory factors.

**Nickel chaperones in enzyme maturation**

One strategy organisms employ to avoid the cytotoxic effects of nickel is the use of metal chaperone proteins, which deliberately guide nickel ions into the catalytic sites of nickel enzymes. Nickel chaperones may also play a role in preventing mismetallation, a term that describes the loading of non-cognate metals. Nickel chaperones would prevent mismetallation if they selectively deliver nickel ions into nickel proteins, while withholding other metals. Analysis of several nickel enzyme maturation systems has led to mechanistic insights, with a recent focus on the metallochaperones that are also NTPases (Table 1). These factors bind and hydrolyze the nucleotide triphosphate molecules ATP or GTP, and there is evidence that the nucleotide state of the chaperone protein plays a regulatory role by affecting activities such as protein-protein interactions and/or nickel-binding [12-15]. In no system, however, has the role of NTP hydrolysis in nickel enzyme maturation been precisely defined.
Table 1. NTPase nickel chaperones in nickel enzyme maturation. An NTPase is frequently involved in nickel enzyme maturation. This research focuses on the nickel chaperone HypB, the NTPase component of [NiFe]-hydrogenase biosynthesis.

<table>
<thead>
<tr>
<th>Ni(II) Enzyme</th>
<th>Chaperone</th>
<th>Chaperone Ni(II) site</th>
<th>NTPase type</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>[NiFe]-hydrogenase</td>
<td>HypB</td>
<td>(Cys)$_4$</td>
<td>P-loop GTPase</td>
<td>[16,17]</td>
</tr>
<tr>
<td>Carbon monoxide dehydrogenase</td>
<td>CooC</td>
<td>(Cys)$_4$</td>
<td>P-loop ATPase</td>
<td>[18,19]</td>
</tr>
<tr>
<td>Urease</td>
<td>UreG</td>
<td>(Cys)$_2$(His)$_2$</td>
<td>P-loop GTPase</td>
<td>[17,20]</td>
</tr>
<tr>
<td>Acetyl-CoA synthase</td>
<td>AcsF</td>
<td>(Cys)$_4$</td>
<td>MinD ATPase</td>
<td>[15]</td>
</tr>
</tbody>
</table>

[NiFe]-hydrogenase maturation

[NiFe]-hydrogenase reversibly catalyzes the production of hydrogen gas from protons and electrons at a bimetallic active site (Figure 1) [21]. One of the final steps in [NiFe]-hydrogenase maturation is nickel insertion, which requires HypA, the GTPase HypB, and in some organisms SlyD (Figure 2) [21,22]. Each of these accessory proteins can independently bind nickel, but together they form multi-protein complexes that likely cooperate during nickel delivery [23].
Figure 1. [NiFe]-hydrogenase and its active site. [NiFe]-hydrogenase catalyzes the reversible reduction of protons to form hydrogen gas. It does so at a complicated bimetallic site that includes Ni(II) bridged by cysteine residues to an Fe(CO)(CN)$_2$ cofactor. The active site is in the large-subunit of [NiFe]-hydrogenase, which is docked against a small subunit that includes three iron-sulphur clusters. In the structure above, of Desulfovibrio gigas [NiFe]-hydrogenase (PDB 2FRV), the active site and iron-sulphur clusters have been highlighted.
Figure 2. Model of [NiFe]-hydrogenase maturation. In *E. coli*, nickel ions (represented as green orbs) are imported into the cytoplasm by the ABC-type primary transporter NikABCDE. Nickel might be received by the transporter as a chelate, which is represented in the figure as an orb in brackets. HypB binds the nickel ion after it is imported, and delivers it to HypA. *Escherichia coli* HypB has two nickel-binding sites; in this figure, nickel is received by a HypB homodimer in the G-domain site, and the N-terminal nickel ions are represented as pale green circles. HypA, which has a structural zinc site represented as a gray circle, delivers nickel to the apo-hydrogenase enzyme, after the enzyme has already received the FeCO(CN)_2 component.

The role of HypB

*Escherichia coli* HypB binds nickel at two sites, a high-affinity site at the N-terminus (K_D = 1.3 ± 0.2 × 10^{-13} M) and a site in the GTP-hydrolyzing domain, called the
G-domain site ($K_D = 1.2 \pm 0.2 \times 10^{-5} M$) [24]. While both binding sites are essential for [NiFe]-hydrogenase maturation in *E. coli* [25], only the G-domain nickel site is common across all HypB homologues in bacteria. This work focuses on the role of the G-domain nickel site in *E. coli* HypB.

In HypB there is close communication between the G-domain nickel site and the nucleotide-binding site in HypB. For instance, the rate of GTP hydrolysis by HypB depends on the metal state of the G-domain [16,26,27]. The connection between metal-binding and GTP hydrolysis is also metal selective, as Ni(II) and Zn(II) inhibit GTP hydrolysis to different degrees, and metal selectivity is potentially allowed by the distinct coordination geometries of the metals in HypB (Figure 3) [16,28]. The connection between the G-domain metal-binding site and the nucleotide-binding site also proceeds in the other direction, as GDP weakens the affinity of HypB for nickel compared to the GTP-loaded protein [13]. In the GDP-loaded state HypB forms a complex with HypA, and nickel (but not zinc) can then be transferred from HypB to HypA (Figure 4) [12,13]. Once HypA receives nickel it no longer forms a complex with HypB [12]. Thus, in this system, the cycling of GTP and GDP is related to the unidirectional and selective transfer of nickel from HypB to HypA. HypA interacts directly with the hydrogenase precursor protein [23,29], so it is likely that HypA proceeds to deliver nickel into the enzyme active site.
**Figure 3.** Nickel and zinc binding by HypB. (a) *Helicobacter pylori* was crystalized in the GDP/P\(_i\) form as a homodimer with nickel in the G-domain binding site (PDB 4LPS). Nickel recruits two cysteine ligands from each member of the dimer. (b) *Methanocaldococcus janaschii* HypB was crystalized in the GTP form as a homodimer with two zinc ions in the G-domain binding site (PDB 2HF8). Three ligands coordinate each zinc ion, including a bridging cysteine, and for one zinc ion a histidine ligand is recruited. Unlike *E. coli* HypB, neither *H. pylori* HypB nor *M. janaschii* HypB have N-terminal nickel-binding sites. C142 in the *H. pylori* crystal (a), and C127 in the *M. janaschii* crystal (b) are homologous to C198 in *E. coli* HypB, the residue studied in this work.
Figure 4. The role of HypB in the maturation of [NiFe]-hydrogenase. According to the current model, the GTPase HypB binds Ni(II) at the interface of a homodimer, and in the GDP state rapidly transfers nickel to HypA. HypB and HypA are known to form a complex, and a transient complex of the two proteins must occur for nickel handoff; however, at this time it is not known whether the transient complex is HypB-HypA or (HypB)$_2$-HypA. HypA can bind to the [NiFe]-hydrogenase large subunit to deliver nickel.

Goals of this project

GTP-GDP cycling by HypB plays an essential role in [NiFe]-hydrogenase maturation. It is clear that communication occurs between the metal-binding and nucleotide-binding domains of HypB; however, how this communication occurs, and the role of this communication in [NiFe]-hydrogenase maturation, has not been clearly defined. It was previously noticed that one of the metal-binding residues in the HypB G-domain nickel-binding site (Cys-142 in H. pylori HypB or Cys-198 in E. coli HypB) is also a member of the Switch II GTPase motif (Figure 5), which is critical for sensing the
GTP-GDP transition [30]. It was therefore proposed that communication between
the metal-binding domain and the nucleotide-binding domain occurs precisely through this
residue [16]. In the *H. pylori* system there is evidence that this is the case, as Ni(II) and
Zn(II) inhibition of GTP turnover, $k_{\text{cat}}$, occurs to a lesser degree in a C142A *HpHypB*
mutant [16]. The other Michaelis-Menten kinetic parameters, $K_M$ and $k_{\text{cat}}/K_M$, were not
determined, nor was the ultimate effect on the biosynthesis of *H. pylori* nickel enzymes
[16].

**Figure 5.** Sequence alignment of *Helicobacter pylori* and *Escherichia coli*
HypB. The alignment was generated by T-Coffee and formatted by
Boxshade. Identical components are highlighted in black, similar
components in gray, and non-similar components in white. In blue is the
conserved Switch II motif, which contains the residue of interest to this
work, C198 in *E. coli* HypB, C142 in the *H. pylori* homologue.
The purpose of this project is to explore the connection between metal-binding and GTP hydrolysis in *E. coli* HypB, and to determine what role this connection plays in [NiFe]-hydrogenase biosynthesis. To this end, the C198T mutant was created in the *E. coli* system and was expected to have an effect *in vitro* similar the effect of C142A in *HpHypB* (Figure 6). It was found that C198T HypB is entirely unable to contribute to [NiFe]-hydrogenase maturation. The biochemical attributes of C198T HypB were explored in an effort to determine why the mutant cannot mature [NiFe]-hydrogenase, and in particular, to determine whether the ultimate cause was decoupling of the nucleotide-binding and nickel-binding sites.

![Diagram](image)

**Figure 6.** Predicted effect of the C198T mutation in HypB. The C198T mutation was made in an effort to decouple metal-binding and GTP hydrolysis, which is inhibited by Ni(II), and strongly inhibited by Zn(II) in the wild-type protein.

‡ “HypB” denotes *E. coli* HypB for the remainder of this work.
Materials and methods

Materials

BL21DE3 and NEBTurbo cell strains were purchased from New England Biolabs, and plasmid purification was performed using the Thermo Fisher GeneJET plasmid miniprep kit. Reagents 2-mercaptoethanol, ammonium molybdate, benzyl viologen, DTNB, EDTA, GDP, Gpp(NH)p, GTP, magnesium chloride hexahydrate, malachite green, NiSO₄ (≥ 99.99 % trace metal basis), PAR, PMB, sodium citrate, sodium dithionate, sodium formate, sodium molybdate, sodium selenite, tween 20, and ZnSO₄ (≥ 99.999 % trace metal basis) were purchased from Sigma Aldrich. Arabinose, DTT, glycerol, GuHCl, Heps, kanamycin, KH₂PO₄, LB, Na₂HPO₄, PMSF, potassium chloride, potassium phosphate, skim milk powder, Tris, tryptone, and yeast extract were purchased from BioShop. PD-10 and Superdex 200 increase 10/300 gel filtration columns were purchased from GE Healthcare. The gel filtration standard, Chelex 100 resin, and goat anti-rabbit HRP conjugate IgG were purchased from BioRad. Sterilizing filters and immobilon-P membrane were purchased from Millipore, as was the Milli-Q filtration system used to prepare 18.2 MΩ.cm resistance water. Milli-Q filtered water was used in all experiments. Anti-HypB polyclonal rabbit antibodies, which were raised against purified HypB protein, were purchased from Cederlane Labs, Burlington, Canada. The SuperSignal West Pico Chemiluminescence BCA reagent, bovine serum albumin standard, and Western-blot detection mixture was purchased from Pierce. Ampicillin was purchased from Bio Basic Canada Inc.
**Plasmid construction**

Primers from Integrated DNA Technologies were used with the Agilent QuikChange mutagenesis kit, for site-directed mutagenesis of hypB-pET24b or hypB-pBAD24 plasmids [24,25]. Both pET24b and pBAD24 vectors containing wild-type hypB, and hypAstr were developed previously, and were used without modification [23,24,31]. The C198T mutation was inserted into hypB on a pET24b vector by Solveig Hasselwander, and on a pBAD24 vector by myself, using QuikChange mutagenesis. The primers used in both mutagenesis experiments are listed in Table 2.

NEBTurbo *E. coli* cells were transformed with the PCR product, and these strains were used to produce hypB plasmid stocks using a GeneJET plasmid miniprep kit. DNA sequencing (ACGT, Toronto, Ontario) was used to confirm that the correct mutation occurred.

| Forward 5'-GTTGGCAACCTCGTAACCCCGGCCAGCTTCGATCTC-3' |
| Reverse 3'-GAGATCGAAGCTGGCAGGGTTACGAGGTTGCAA-5' |

**Table 2.** Primers used to create C198T hypB from wild-type hypB. The mutation was introduced to hypB on both pET24b and pBAD24 vectors.

**Protein expression and purification**

HypAstr, wild-type HypB, and C198T HypB were overexpressed in BL21DE3 *E. coli* and purified as previously described [13,24]. In the case of HypB, the protein was purified and stored in Buffer A. In the case of HypAstr, the protein was purified and
stored in Buffer B. The components of these buffers, and of other important buffers used in this work, are listed in Table 3.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Components</th>
</tr>
</thead>
</table>
| Buffer A | 25 mM Hepes (pH 7.5)  
200 mM NaCl  
1 mM TCEP |
| Buffer B | 50 mM Tris (pH 7.5)  
500 mM NaCl  
10 % glycerol  
1 mM TCEP |
| Buffer C | 25 mM Hepes (pH 7.5)  
200 mM KCl  
2.5 % glycerol |
| Buffer D | 25 mM Hepes (pH 7.5)  
200 mM KCl  
2.5 % glycerol  
5 mM MgCl₂ |
| Buffer E | 25 mM Hepes (pH 7.5)  
200 mM KCl  
2.5 % glycerol  
5 mM MgCl₂  
1 mM TCEP |
| Buffer F | 25 mM Hepes (pH 7.5)  
200 mM KCl  
5 mM MgCl₂  
1 mM TCEP |

Table 3. Important buffers used in this work, and their components.

The concentration of purified protein was determined by electronic absorbance spectroscopy, using the 280 nm absorption, following 2 hr incubation with 250 mM EDTA. The concentration was calculated using the extinction coefficient of 16,500 M⁻¹ cm⁻¹ at 280 nm for the HypB proteins, and 17,990 M⁻¹ cm⁻¹ at 280 nm for HypAstr [13].
A PAR assay was used to determine the metal content of purified proteins. Proteins were incubated in a buffered solution (250 mM Tris, pH 8.5) that contained 4 M GuHCl, 1.8 mM PMB, and 450 µM PAR. The absorbance at 500 nm corresponded to formation of (PAR)$_2$Me(II), where Me(II) was either Zn(II) or Ni(II). The 500 nm signal was compared against a standard curve prepared under the same conditions, of NiSO$_4$ in the case of HypB, and ZnSO$_4$ in the case of HypA$_{str}$. HypB was typically purified with ~1 equivalent of metal bound to the high affinity nickel site at the N-terminus of E. coli HypB. HypA$_{str}$ was typically purified with ~1 equivalent of metal bound to a high affinity zinc site. The nickel content of HypB was also determined by monitoring the absorbance at 320 nm of HypB, as a LMCT occurs ($\varepsilon = 7,300$ M$^{-1}$ cm$^{-1}$) when the protein is purified with nickel in the N-terminal site [24]. The concentration of nickel bound to the HypB N-terminal could therefore be compared against the concentration of total HypB, to determine the percent of holo-HypB.

The free-thiol content of the purified proteins was determined by incubating them with DTNB, which produces 5-mercapto 2-nitrobenzoic acid on reaction with the sulphhydryl groups of cysteine residues. Purified protein was desalted in a glove box, under anaerobic atmosphere (95 % N$_2$, 5 % H$_2$), using a PD-10 column equilibrated with a solution of 25 mM Hepes (pH 7.5) and 200 mM NaCl in order to remove TCEP. The desalted protein was incubated with 6 M GuHCl, 10 mM EDTA, and 18 – 20 mM DTNB. The free-thiol content was determined by comparing the absorbance of the protein-DTNB mixture at 412 nm against a standard curve of 2-mercaptoethanol that was prepared under the same conditions.
The purified protein was analyzed by ESI-MS, and compared against its theoretical mass (Table 4). In the case of HypB, this corresponds to the product of the *hypB* gene, minus the N-terminal methionine residue, which is lost post-translationally. Purified HypB and HypA*str* were usually observed by ESI-MS to be within 1 Da of the theoretical mass.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Predicted average mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type HypB</td>
<td>31,433.8 Da</td>
</tr>
<tr>
<td>C198T HypB</td>
<td>31,431.8 Da</td>
</tr>
<tr>
<td>HypA<em>str</em></td>
<td>14,208.3 Da</td>
</tr>
</tbody>
</table>

**Table 4.** The average mass of proteins used in this work. HypB is purified without the N-terminal methionine residue, which is removed in *E. coli*.

*Nickel affinity experiments*

Purified protein was exchanged into TCEP-free buffer (Buffer C, Table 3) within an anaerobic glove box by using a PD-10 column. The buffer had been stirred with 0.2% (w/v) Chelex 100 resin for 1 hr, before Chelex 100 resin was filtered off, and the buffer was stirred overnight under anaerobic atmosphere to eliminate dissolved oxygen. Samples of 10 μM HypB or 10 μM C198T HypB with 0 – 100 μM Ni(II) were incubated overnight in the glove box at 4 °C. A LMCT arises at 340 nm when nickel is in the G-domain site. Absorbance values were converted into fractional saturation (Equation 1).
Equation 1. Determining fractional saturation from absorbance. The portion of proteins saturated with nickel in the G-domain (r) was determined from the absorbance at 340 nm ($A_{obs}$) using the minimum absorbance at 340 nm ($A_{min}$), as well as the absorbance at 340 nm upon saturation with nickel ($A_{max}$).

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

Fractional saturation, r, was plotted against free nickel (Equation 2).

\[ [\text{Ni(II)}]_{\text{free}} = [\text{Ni(II)}]_{\text{total}} - r[\text{HypB}]_{\text{total}} \]

Equation 2. Determining the concentration of free nickel. The concentration of free nickel was found using the total concentration of nickel in each sample, the fractional saturation of HypB (Equation 1), and the total concentration of HypB. A monomer binding model was used, and the value inputted for $[\text{HypB}]_{\text{total}}$ reflected the total concentration of HypB monomeric units in solution.

This plot was fit to the Hill equation to determine the apparent dissociation constant (Equation 3).
Equation 3. The Hill equation. $K_D$ is the apparent dissociation constant, $n$ is the Hill coefficient, and $r$ is the fractional saturation of HypB (Equation 1).

The guanine nucleotide signal at 253 nm tended to interfere with the comparatively weak LMCT band at 340 nm, caused by nickel in the G-domain. Also, a LMCT band does not arise on zinc-binding to the G-domain, due to the full d-shell of Zn(II). For these reasons mag fura 2 (MF2), a fluorescent competitor of Ni(II) and Zn(II), was used to find metal $K_D$ in the presence of nucleotides. In these experiments HypB was exchanged into TCEP-free buffer (Buffer D, Table 3) by spin concentration using a 0.5 mL 10 kDa centrifugal filtration unit (Sigma Aldrich). The buffer and the nucleotide stocks were treated with 0.2 % (w/v) Chelex 100 resin (BioRad), for 1 hr, and the Chelex 100 resin was removed before adding 5 mM MgCl$_2$. The buffer was stirred overnight under an anaerobic atmosphere in a glove box to remove dissolved oxygen. Either 20 µM or 40 µM HypB was incubated overnight in the glove box with 1.0 µM MF2, 500 µM GDP or 500 µM Gpp(NH)p, and 0 – 1000 µM metal-sulphate (NiSO$_4$ or ZnSO$_4$). After overnight incubation, the fluorescent emission of MF2 at 500 nm ($\lambda_{ex} = 335$ nm) was measured, and converted to fractional saturation (Equation 4).
\[ r = 1 - \frac{F_{\text{obs}} - F_{\text{min}}}{F_{\text{max}} - F_{\text{min}}} \]

**Equation 4.** Determining fractional saturation from the fluorescent intensity of mag fura 2. The portion of MF2 saturated with nickel (r) was determined from the fluorescent intensity at 500 nm (\( F_{\text{obs}} \)), the maximum fluorescent intensity at 500 nm (\( F_{\text{max}} \)) as well as the fluorescent intensity at 500 nm upon saturation with nickel (\( F_{\text{min}} \)). The fraction is subtracted from one since saturation of MF2 with nickel corresponds to the minimum fluorescent intensity. The excitation wavelength was 335 nm.

Fractional saturation of MF2 was plotted against \([\text{Ni(II)}]_{\text{free}}\) or \([\text{Zn(II)}]_{\text{free}}\), and fit to the Hill equation (Equation 3). From this analysis it was determined that MF2 has an apparent dissociation constant of 2.0 ± 0.7 µM for Ni(II), and 0.9 ± 0.2 µM for Zn(II). The fractional saturation of MF2 in samples that contained HypB was plotted against \([\text{Ni(II)}]_{\text{total}}\) or \([\text{Zn(II)}]_{\text{total}}\), and fit to a monomer binding model, using a custom DynaFit script (Figure A5).

Zinc-binding to C198T HypB in the absence of nucleotide was also measured by another competition experiment, using a Cintra 404 UV-visible spectrometer (GBC). In this experiment, Zn(II) competed with Ni(II) for the G-domain binding site of C198T HypB, and loss of the 340 nm LMCT occurred when Ni(II) was replaced with Zn(II). The electronic absorption spectrum was measured between 250 nm and 500 nm for separate samples, which included 1 µM C198T HypB, 120 µM Ni(II), and 0 – 8 µM Zn(II) in Buffer C (Table 3). Samples were prepared under the anaerobic atmosphere, and were
allowed to incubate at 4 °C in the glovebox overnight. The absorbance of samples at 340 nm was recorded in a cuvette with 10 cm path length, and converted to fractional saturation with nickel (Equation 1). Fractional saturation vs. [Zn(II)]_{total} were fit using a custom DynaFit script (Figure A6), using a nickel dissociation constant of 40 µM.

Circular dichroism spectroscopy
HypB was exchanged under anaerobic atmosphere into 100 mM potassium phosphate buffer (pH 7.5) using two PD-10 columns in series. The CD spectrum between 180 – 260 nm was measured with 40 µM protein at 6 °C (70 increments, 2 s integration, average of 3 scans) by a Jasco J-170 spectropolarimeter with 1 mm path length. The mean residue ellipticity (Equation 5 [32]) was plotted against wavelength.

\[
[\theta]_{\text{mre},\lambda} = \frac{(\frac{\text{MM}}{N-1}) \theta_\lambda}{[\text{protein}] \times 1 \times 10}
\]

**Equation 5.** Mean residue ellipticity. \(\theta_\lambda\) is ellipticity at wavelength \(\lambda\) (deg), \([\theta]_{\text{mre}}\) is mean residue ellipticity at wavelength \(\lambda\) (deg cm\(^2\) dmol\(^{-1}\)), 1 is path length (cm), and [protein] is in units of g/mL. The MM/N-1 factor calculates the mean residue weight. MM is the molecular weight of the protein in Da, N is the number of amino acids, and N-1 is the number of peptide bonds.

To conduct a thermal denaturation experiment the temperature of the sample was ramped between 10 °C and 85 °C in increments of 5 °C, with 1 min equilibration time at each temperature. Spectra were collected as single scans, rather than in triplicate.
[\theta]_{mre} across the second band in the CD spectrum (218 – 224 nm) was averaged at each temperature increment. A two-state model was used in the calculation of \( T_m \); at high-temperature the protein was described as “unfolded”, and at low temperature it was described as “folded”. The temperature was plotted against \( \alpha \), the fraction of folded protein (Equation 6). The point where \( \alpha = 0.5 \) (\( T_m \)) was identified by fitting the data to a sigmoidal curve of variable slope using Prism software.

\[
\alpha = \frac{[\theta]_{mre,i} - [\theta]_{mre,U}}{[\theta]_{mre,F} - [\theta]_{mre,U}}
\]

**Equation 6.** The fraction protein that is folded. \([\theta]_{mre}\) is the average value of the mean-residue-ellipticity from 218 – 224 nm for any given temperature (i), in the unfolded state (U), or the folded state (F).

The CD spectrum of 40 \( \mu \)M HypB in the presence of 0 – 100 \( \mu \)M Ni(II) was also measured. In this case, the buffer used was a diluted Hepes buffer (2.5 mM Hepes pH 7.5, 20 mM KCl), rather than 100 mM potassium phosphate buffer, as the solubility of nickel phosphate is very low in water [33], and at high concentrations Hepes absorbs strongly at and below 200 nm in CD spectroscopy [32].

*Hydrogenase assay*

*Preperation of cell lysates*

This experiment used MC4100 strain *E. coli*, a hypB knockout (\( \Delta \text{hypB} \)) MC4100 strain [34], as well as \( \Delta \text{hypB} \) MC4100 transformed with *wild-type hypB*-pBAD24 or *C198T hypB*-pBAD24. A single colony of *E. coli* was used to inoculate 10 mL LB media
(0.01% ampicillin), which was shaken at 37 °C for 8 hr to prepare a pre-culture. TYET media (Table 5) was used to fill a 50 mL falcon tube to the brim (~57 mL). This TYET culture was inoculated with 400 µL aerobic pre-culture, before it was sealed tightly and shaken overnight at 37 °C to induce anaerobic metabolism. Tris buffered media was used instead of the more common phosphate buffered media (TYEP) because nickel-phosphate is insoluble in water [33].

<table>
<thead>
<tr>
<th>Supplemented TYET media</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Component</td>
<td></td>
</tr>
<tr>
<td>Tryptone</td>
<td>1 %</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.5 %</td>
</tr>
<tr>
<td>Tris</td>
<td>50 mM (pH 7.5)</td>
</tr>
<tr>
<td>Sodium formate</td>
<td>30 mM</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.8 %</td>
</tr>
<tr>
<td>Arabinose</td>
<td>10 µM</td>
</tr>
<tr>
<td>The above components were stirred with 0.2 % (w/v) Chelex 100 resin for 1 hr before the resin was removed by vacuum filtration, and the media completed by addition of metals and antibiotic.</td>
<td></td>
</tr>
<tr>
<td>Sodium molybdate</td>
<td>1 µM</td>
</tr>
<tr>
<td>Sodium selenite</td>
<td>1 µM</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>0.01 %</td>
</tr>
<tr>
<td>Completed media was passed through a sterilizing 0.2 µm filter and used fresh.</td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Protocol for making supplemented TYET media. The media was made in two stages. First, non-metallic components and water were mixed with Chelex 100 resin for 1 hr. Second, Chelex 100 resin was removed and the metal nutrients molybdate and selenite were added. Ampicillin was added at this stage as well. The final mixture was passed through a 0.2 µm sterilizing filter, into a sterile bottle, and used fresh.
After overnight growth the anaerobic cultures were pelleted by centrifugation (30 min, 4000 rpm, 4 °C). The pellet was suspended in 2 mL ice-cold 40 mM potassium phosphate buffer (KPB, pH 7.6), and centrifuged to form a washed pellet (30 min, 4000 rpm, 4 °C). This was suspended in 400 µL ice-cold solution of 50 mM KPB (pH 7.6), 200 µM PMSF, and 1 mM DTT. These samples were lysed using the Branson Sonifier Cell Disruptor 185, which was run at low power (output No. 3) for three 10 s intervals, with > 10 min of cooling in an ice bath between the 10 s sonication steps. The lysed cells were centrifuged (14,800 rpm, 20 min, 4 °C) to pellet the cell debris. The supernatants were frozen in liquid nitrogen and stored at -80 °C until use in the hydrogenase assay or for Western blotting.

**Determination of lysate activity**

The hydrogenase assay reagents, 4 mM benzyl viologen in KPB, and 10 mM sodium dithionite in KPB, were prepared under an anaerobic atmosphere in a glove box. Septum sealed cuvettes containing 1.5 mL aliquots of benzyl viologen were removed from the glove box along with a 3 mL solution of sodium dithionite in a septum sealed vial. Sodium dithionite solution was injected into the benzyl viologen solution until the solution remained purple after mixing (~10 µL), which indicates that it is reducing and that the oxygen has been removed. Between 5 µL and 20 µL cell lysate was injected into the cuvette to start the reaction, and the injected volume was accurately recorded. The absorbance at 600 nm was measured every 5 s for a total of 150 s after mixing by inversion. The specific activity was calculated from the slope (Equation 7).
Specific activity = \( \frac{\text{slope} \times 60 \, \text{s} \, \text{min}^{-1} \times 1.5 \times 10^{-3} \, \text{L} \times 10^6 \, \text{µmol} \, \text{mol}^{-1}}{7,400 \, \text{M}^{-1} \, \text{cm}^{-1}} \)

**Equation 7.** Specific activity. Specific activity is in units of µM/min, while slope is in units of AU/s. The 7,400 M\(^{-1}\) cm\(^{-1}\) factor is the molar extinction coefficient for reduced benzyl viologen.

Specific activity was converted into activity (µmol/min•mg) using the known volume of lysate added and total protein concentration of the lysate (Equation 8).

\[
\text{Activity} = \frac{\text{(specific activity)}}{[\text{protein}] \times 10^{-3} \, \text{mL} \, \text{µL}^{-1} \times (\text{µL} \, \text{lysate})}
\]

**Equation 8.** Hydrogenase activity. Activity is in µmol/min•mg, [protein] is the total protein concentration in mg/mL, and “µL lysate” is volume of lysate added to the cuvette of benzyl viologen solution.

The total protein content was determined by a BCA assay using a standard of bovine serum albumin.

*Western blot*

Immediately after thawing the lysate on ice, the lysate contents were resolved on a 12.5 % SDS-polyacrylamide gel (160 V, 50 min, 4 °C), after the gel was already run without any material loaded (160 V, 50 min, 4 °C). The contents of the lysate, resolved by SDS-PAGE, were transferred to an Immobilon-P membrane (400 mA, 60 min, 4 °C)
that had been activated by 2 min incubation in methanol and pre-equilibrated with transfer buffer (150 mM glycine, 25 mM Tris (pH 8.0), 20 % (v/v) methanol). The membrane was blocked in 5 % (w/v) skim-milk powder in PBST buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 0.05 % (v/v) Tween 20, pH 7.4). After rinsing in PBST buffer the membrane was probed with anti-HypB polyclonal rabbit antibody (Cedarlane Labs, Burlington, Canada) at 1:1000 dilution, for 1 hr in PBST at room temperature. After further rinsing, the blot was probed with goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase, at 1:30,000 dilution in PBST, for 30 min at room temperature. The secondary antibody was detected on membranes rinsed with PBST by chemiluminescence, using the SuperSignal West Pico Chemiluminescence detection mixture.

*Malachite green assay of GTPase activity*

Buffer D (Table 3) was stirred with 0.2 % (w/v) Chelex 100 resin for 1 hr, and Chelex 100 resin was removed by vacuum filtration before addition of Mg(II), and in some experiments Ni(II) or Zn(II). This buffer was stirred overnight in the glove box, under anaerobic atmosphere, so that the solution was anaerobic. All other solutions were prepared in this buffer, and protein was exchanged into it using a PD 10 column. After the reaction mixtures were prepared, they were pre-warmed to 37 °C, and the kinetic experiment was initiated by addition of HypB. The final solutions had 160 µL total volume, contained 2 – 4 µM HypB, and GTP content scaled between 0 – 550 µM. HypB was allowed to react with GTP for 20 min at 37 °C under anaerobic atmosphere. After 20 min, 40 µL malachite green detection mixture was added (12 % H₂SO₄ (ACP), 0.08 % malachite green, 1.5 % ammonium molybdate, 0.17 % Tween 20). The colour was
allowed to develop for 5 min, before addition of 23 µL 34 % sodium citrate to stabilize the malachite green-phosphomolybdate complex. The absorbance of the malachite green-phosphomolybdate complex at 630 nm was compared against a standard curve of inorganic phosphate (P$_i$) prepared using the same conditions. The results were used to find the amount of P$_i$ in each sample, which could be converted to velocity by dividing the amount P$_i$ by the time of incubation. Velocity vs. amount GTP were modeled by Michaelis-Menten kinetics (Equation 9).

\[ v = \frac{V_{\text{max}}[S]}{K_M + [S]} \]

**Equation 9.** Michaelis-Menten kinetics. $V_{\text{max}}$ is the maximum velocity, $v$ is velocity at any given concentration of the substrate [S] which for these experiments was GTP, and $K_M$ the Michaelis-Menten constant.

Before determining the Michaelis-Menten kinetic parameters, 4 µM HypB was shown to be at initial velocity for 200 min at 37°C, with 500 µM GTP (data not shown).

**Size exclusion chromatography**

The effect of nucleotide and metal on HypB quaternary structure, and the formation of HypB-HypA$_{str}$ complex, was investigated by size exclusion chromatography. A Superdex 200 Increase 10/300 was equilibrated with two volume equivalents of reducing buffer (Buffer E, Table 3). Depending on the desired sample conditions, the buffer also contained 100 µM NiSO$_4$ and/or 100 µM GDP. Protein elution was monitored by absorbance at 280 nm and compared against a gel filtration molecular
weight standard. Some early experiments, discussed in this work, were performed with glycerol-free buffer (Buffer F, Table 3).

**Results and discussion**

The C198T mutation in *E. coli* HypB was intended to be analogous to the C142A mutation in *HpHypB*, which reduced the inhibition of GTPase activity that is caused by both Zn(II) and Ni(II) [16]. The aim was to investigate whether the analogous residue in *E. coli* HypB also served as a link between the metal binding and GTPase activities in this protein, and if so, to determine the impact of decoupling these activities on hydrogenase production. The threonine mutation is the third substitution attempted at this position in *E. coli* HypB, and was preceded by work on C198A and C198S mutants of HypB by undergraduate students in the lab, under the supervision of Colin Douglas. In the case of C198S HypB, Solveig Hasselwander could not purify the mutant protein using the standard HypB purification protocol (unpublished data, 2014). In the case of C198A HypB, Rohan Ravindranath purified the protein, however observed by CD spectroscopy that nickel caused the protein to unfold (unpublished data, 2013). It was thought that a stable substitution might require a more bulky amino acid at the C198 position than serine or alanine, so Solveig Hasselwander and Colin Douglas proposed a threonine substitution, and created the *C198T hypB*-pET24b vector used in this work. It is important to note that the C198T substitution is not structurally conservative, because the methyl group of threonine is a hydrophobic moiety absent from cysteine, and therefore not normally found in the HypB G-domain nickel-binding site. Unlike the previous C198 substitutions, the C198T mutation did not prevent expression and purification of HypB, nor did it perturb HypB secondary structure (see sections *Purification of C198T HypB*).
This Results and discussion section begins with an examination of the effect of the C198T mutation on hydrogenase production in vivo.

*C198T HypB does not traffic nickel to [NiFe]-hydrogenase*

Although ΔhypB E. coli produces no active hydrogenase, hydrogenase activity can be partially restored when nickel is added to the growth media [35]. HypB was assigned a role in nickel trafficking in part because of these observations, which are reproduced in the present work (Figure 7). To determine whether C198T HypB could also function in nickel trafficking, the hydrogenase activity of lysate from cells expressing C198T HypB was measured (Figure 7). While ΔhypB E. coli that express wild-type HypB produce hydrogenase, expression of C198T HypB does not recover any activity. When 800 µM Ni(II) is added to the growth media, some activity is recovered in the C198T HypB expressing strain, although the level of activity remains the same as the ΔhypB strain, and is significantly less than the strain that expresses wild-type HypB. Together these data indicate that C198T HypB does not function in nickel trafficking and cannot contribute to the maturation of [NiFe]-hydrogenase.
**Figure 7.** The hydrogenase activity of *E. coli* expressing C198T HypB. Hydrogenase activity was assessed by adding cell lysate to a solution that contained benzyl viologen, a redox active dye. In this assay no activity is observed in the lysate of the Δ*hypB* strain, and activity is restored if wild-type HypB is expressed in that strain. In contrast, expression of C198T HypB does not recover any activity. These data are the averages of three biological replicates, and error bars represent ± 1 standard deviation.

C198T HypB would not traffic nickel if the protein was improperly expressed or degraded in the cell. To test this possibility, Western analysis was used. C198T HypB is expressed from plasmid pBAD24 at levels similar to that of wild-type HypB (Figure 8, Figure 9). It can be concluded, therefore, that impaired expression or degradation of
C198T HypB within the cell does not explain the impact of the mutation on hydrogenase activity.

Figure 8. Western analysis of C198T HypB expression in *E. coli* grown in media with 800 µM nickel. The reduced hydrogenase activity in *C198T hypB*-pBAD24 strain is due to neither degradation of C198T HypB nor poor protein expression off of the vector. The level of expression in the C198T HypB strain is similar to the level of wild-type HypB expressed in the strain that contained *wild-type hypB*-pBAD24.
Figure 9. Western analysis of C198T HypB expression in *E. coli* grown in media with no nickel added. While the *C198T hypB*-pBAD24 strain does not have any activity, this is neither due to degradation of C198T HypB, nor to poor protein expression. The level of expression in the C198T HypB strain is similar to that observed in the strain that contained *wild-type hypB*-pBAD24.

C198T HypB was purified in an effort to establish the functional consequences of the mutation on the biochemistry of the protein. If C198T HypB was found *in vitro* to have poor communication between the nickel-binding site and the nucleotide-binding site, *in vitro* and *in vivo* data together might illustrate the importance of this communication to the maturation of [NiFe]-hydrogenase. The data discussed below show that, unexpectedly, communication between the HypB nucleotide-binding and metal-binding sites was maintained to a large degree, in spite of the C198T mutation. Further biochemical detective work was therefore conducted in order to tease out the precise functional consequences of the C198T mutation.
Purification of C198T HypB

C198T HypB was purified by the usual HypB purification protocol [24], and ESI-MS consistently showed the purification product to be within 1 Da of the expected mass, 31,431.8 Da (Table 4, Figure A1). Therefore, at least in this *E. coli* over-expression system, C198T HypB is produced with the correct primary structure.

Electron absorption spectroscopy showed that C198T HypB had, without the addition of any nickel to the purification buffer, an absorption band at 320 nm that is consistent with the LMCT caused by nickel in the N-terminal nickel-binding site of HypB (Figure 13) [24]. This result is in agreement with a colorimetric analysis of metal content, which showed that C198T HypB could be purified with $\geq 0.9$ equivalents of metal (data not shown). HypB is normally purified with nickel in the N-terminal site, and the site is essential for hydrogenase maturation in the case of *E. coli* [25]. The N-terminal nickel-binding site was not under study in this thesis work and is not discussed further. The protein was also shown by a colorimetric assay to have been purified with $\geq 90\%$ of its cysteine residues in the reduced form (data not shown). It is essential that C198T HypB be in the reduced state, given that the sulphhydryl groups of reduced cysteine residues are involved in metal coordination at both the N-terminal and G-domain nickel sites. Purified C198T HypB was used in further experiments if it had the correct molecular weight, as determined by mass spectrometry, and met the conditions of being *holo* and reduced.
Secondary structure of C198T HypB

Altered secondary structure or stability could explain why C198T HypB is unable to participate in hydrogenase maturation. However, CD spectroscopy reveals that C198T HypB has a secondary structure that is similar to that of wild-type HypB (Figure 10), as well as a $T_m$ similar to that of wild-type HypB (Figure 11), so misfolding does not explain why C198T HypB is unable to traffic nickel properly. Furthermore, nickel affects the secondary structure of C198T HypB in the same way that it affects the secondary structure of wild-type HypB (Figure A3). The CD samples that were used in the nickel titration experiment contained HypB protein in a dilute Hepes solution (2.5 mM Hepes (pH 7.5), 20 mM KCl, 100 µM TCEP), and the experiment should be repeated using a more strongly buffered system. This would require the use of a buffer such as Tris or borate, which would absorb less strongly than Hepes at and below 200 nm.
**Figure 10.** Circular dichroism spectroscopy of C198T HypB and wild-type HypB. The average of three scans (70 increments, 2 s integration) for samples of 40 µM protein in 100 mM potassium phosphate buffer (pH 7.5) prepared in the glove box under an anaerobic atmosphere.

**Figure 11.** The Tₘ of C198T HypB as measured by CD spectroscopy. The average absorbance at the second CD band (218 – 224 nm, Figure 10) was measured between 6 °C and 85 °C and converted to fractional folding (α, Equation 6). Tₘ, temperature where α = 0.5, was identified by fitting the data to a sigmoidal curve of variable slope using Prism software.
Although C198T HypB cannot traffic nickel, the above data suggest that this loss in function is not caused by protein misfolding at 37 °C, the temperature at which *E. coli* were grown. The ability of C198T HypB to carry out the primary biochemical activities of HypB, GTP hydrolysis, metal-binding, and protein-protein interactions, was characterized at this stage.

*GTPase activity of C198T HypB*

HypB is a GTPase, and this activity is essential for delivery of nickel to [NiFe]-hydrogenase. For instance, when the GTPase activity of HypB is abrogated by mutation, *E. coli* have no active hydrogenase activity, and [NiFe]-hydrogenase is produced without nickel [36]. C198 is not only a metal-binding residue, but also a member of the Switch II GTPase motif, so it is possible that the C198T mutation would affect the ability of C198T HypB to hydrolyze GTP altogether.

Before analyzing the GTPase activity of C198T HypB, the conditions of the GTPase assay were optimized. First, Hepes-KCl buffer was used in the place of Hepes-NaCl buffer, as work with the *H. pylori* homologue demonstrated that potassium improved the turnover of GTP by *HpHypB* [16]. The experiment was also conducted under an anaerobic atmosphere, so that the reducing agent TCEP would not be needed. TCEP can be considered as a non-innocent bystander, as it binds transition metals [37], and experience in the lab indicates that it can affect the apparent *K_D* of *E. coli* HypB for nickel. C198T HypB was found to aggregate at Ni(II) concentrations ≥ 130 µM (data not shown), so 2.5 % (v/v) glycerol was included to help stabilize the protein in buffered
solution. In the wild-type protein, glycerol did not affect the rate of GTP hydrolysis unless the concentration used was at or above 5% (v/v) (Figure A4). Under these conditions the $k_{\text{cat}}$ of GTP hydrolysis for WT HypB was $0.7 \pm 0.3$ min$^{-1}$ (Table 6). This is higher than previously described $k_{\text{cat}}$ values (~0.2 min$^{-1}$ [26,38]), and the difference in activity is likely a result of the different buffer conditions used.

Analysis of C198T HypB revealed that it retained GTPase activity (Figure 12), and the kinetic parameters $k_{\text{cat}}$ and $K_M$ were similar to those of the wild-type protein (Table 6). Therefore, mutating the Switch II residue did not eliminate the ability of the protein to hydrolyze GTP, and the inability of C198T HypB to mature [NiFe]-hydrogenase is not explained by loss of function at this level. The next step was to test whether hydrolysis of GTP by C198T HypB remained metal-sensitive. It was expected that this mutation would decouple the two activities, because an analogous mutation of the H. pylori homologue, HpHypB, diminished the inhibition of Ni(II) and Zn(II) on GTP hydrolysis.

However, in contrast to the study with HpHypB, the GTPase activity of C198T HypB was inhibited by both Ni(II) and Zn(II) to a similar degree as the wild-type E. coli protein. In the presence of 100 µM Ni(II), the $k_{\text{cat}}/K_M$ parameter was significantly reduced, by approximately 50% for C198T HypB, and by approximately 80% for wild type HypB. While it is possible that nickel inhibition is attenuated to some extent by the C198T mutation, this possibility could not be assessed given the degree of error in the data. Surprisingly, nickel inhibition mostly occurred through an increase in $K_M$, rather
than through a reduction of $k_{cat}$, which indicates competitive inhibition of GTPase activity (Table 6). Previous work indicated that Ni(II) inhibits $k_{cat}$ in *E. coli* HypB [26]. The full Michaelis-Menten curves were not described in the previous study, so it is possible that more extensive analysis would have shown that the Ni(II) inhibited samples trend towards the same $V_{max}$ value as samples in metal-free buffer.

One of the implications of this unexpected result is that Ni(II) inhibition of GTPase activity may not be relevant to the biochemistry of HypB *in vivo*. This is because the concentration of the substrate, GTP, is at millimolar levels in *E. coli*, which is much larger than the mid $\mu$M $K_{M}$ value for GTP hydrolysis by HypB [39]. The GTPase activity of HypB will therefore operate at $V_{max}$ in *E. coli* even in the presence of nickel. Zn(II), on the other hand, strongly inhibited $k_{cat}$ for both C198T HypB and wild-type HypB (Figure 12). In the cell, therefore, relevant inhibition of GTP hydrolysis might only occur when HypB binds Zn(II). These results suggest that GTP hydrolysis plays a role in achieving metal selectivity, a process essential to keeping the “Ni” in [NiFe]-hydrogenase.

While these experiments shed some light on the biochemical features of HypB, they do not explain why C198T HypB is unable to function in [NiFe]-hydrogenase maturation. Contrary to the initial prediction (Figure 6), C198T HypB continues to hydrolyze GTP in a metal-dependent and metal-selective manner, much like the wild-type protein.
Table 6. Kinetics of GTP hydrolysis by C198T HypB and wild-type HypB. The $k_{cat}$, $K_M$ and $k_{cat}/K_M$ values are the averages ± 1 standard deviation of 4 – 5 replicate measurements. C198T HypB is an active GTPase, and the kinetics of GTP hydrolysis are similar to that of the wild-type protein. For both C198T HypB and wild-type HypB the efficiency of GTP hydrolysis, $k_{cat}/K_M$ is significantly reduced by 100 µM Ni(II). Unexpectedly, Ni(II) inhibits GTP hydrolysis by increasing $K_M$, rather than decreasing $k_{cat}$.

<table>
<thead>
<tr>
<th></th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$K_M$ (µM)</th>
<th>$k_{cat}/K_M$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type HypB</td>
<td>0.7 ± 0.3</td>
<td>40 ± 10</td>
<td>300 ± 100</td>
</tr>
<tr>
<td>Wild-type HypB + 100 µM Ni(II)</td>
<td>0.8 ± 0.7</td>
<td>180 ± 100</td>
<td>60 ± 50</td>
</tr>
<tr>
<td>C198T HypB</td>
<td>1.8 ± 0.5</td>
<td>120 ± 40</td>
<td>270 ± 70</td>
</tr>
<tr>
<td>C198T HypB + 100 µM Ni(II)</td>
<td>1.3 ± 0.3</td>
<td>180 ± 80</td>
<td>140 ± 50</td>
</tr>
</tbody>
</table>
Figure 12. The effect of zinc on the GTP hydrolysis of wild-type HypB and C198T HypB. (a) GTP hydrolysis by C198T HypB (2 µM) can be fit to Michaelis-Menten kinetics in the absence of metal ($K_M = 120$ µM, $k_{cat} = 1.5$ min$^{-1}$, $k_{cat}/K_M = 200$ M$^{-1}$ s$^{-1}$). (b) When just 20 µM Zn(II) is added to the buffer solution, C198T HypB (2 µM) is no longer an active GTPase. (c) GTP hydrolysis by wild-type HypB (4 µM) in the absence of metal can also be fit to Michaelis-Menten kinetics ($K_M = 43$ µM, $k_{cat} = 0.63$ min$^{-1}$, $k_{cat}/K_M = 240$ M$^{-1}$ s$^{-1}$). (d) When just 20 µM Zn(II) is added to the buffer solution, the $k_{cat}$ of wild-type HypB (4 µM) is reduced. $K_M$ is also much smaller, so the $k_{cat}/K_M$ value is similar to that observed in conditions where no metal was added ($K_M = 17$ µM, $k_{cat} = 0.13$ min$^{-1}$, $k_{cat}/K_M = 130$ M$^{-1}$ s$^{-1}$). Although the concentration of enzyme used (either 2 µM or 4 µM) affects the measured velocity, it does not affect the reported Michaelis-Menten kinetic parameters ($k_{cat}$, $K_M$, and $k_{cat}/K_M$).
In wild-type HypB, communication between the nickel-binding and nucleotide-binding sites goes in both directions, and involves more than metal inhibition of GTPase activity. For instance, communication between the sites also causes the nucleotide state of HypB to affect nickel-binding, as well as the ability of HypB to complete nickel handoff to HypA [12]. Communication from the nucleotide-binding site to the metal-binding site is likely involved in metal specificity. For instance, while nucleotide can affect the $K_D$ for nickel, it does not affect the $K_D$ for zinc [12]. The nucleotide-dependent metal affinity of C198T HypB and wild-type HypB was explored to determine whether the C198T mutation disrupts communication in this direction.

Metal-binding activity of C198T HypB

Nickel-binding is an essential quality of HypB, given that it is a nickel chaperone protein. C198T HypB is missing a metal-ligating cysteine residue and was expected to have reduced affinity for nickel compared to the wild-type protein. To test this prediction, the $K_D$ of C198T HypB bound to Ni(II) was determined, as was the effect of guanine nucleotides GDP and GTP on metal affinity.

Nickel-binding to the G-domain of C198T HypB could be monitored by watching the electronic absorption spectrum, as the cysteine-Ni(II) bond causes a LMCT band at 340 nm (Figure 13). The $K_D$ could be determined under nucleotide-free conditions by tracking this LMCT. For C198T HypB, the dissociation constant for nickel was $40 \pm 10 \mu M$ in the absence of nucleotide, which is approximately $10\times$ weaker than the dissociation constant for wild-type HypB under the same conditions ($K_D = 4 \mu M$, Figure 14), so removal of the cysteine residue did weaken HypB nickel affinity. The best-fit Hill
coefficient values varied a great deal between experiments and were sometimes extremely large. For instance, over the course of four experiments, the Hill coefficient varied between 2 – 7. These values are “unrealistically” high; in the case of hemoglobin, where O₂ binds with high cooperativity, this value only varies between 1.7 to 3.2 [40]. It is difficult to visualize the nickel-binding site on one C198T HypB monomer cooperatively interacting with the nickel-binding site of another C198T HypB monomer, so it is unclear what gives rise to this this sigmoidal Ni(II)-binding curve, and how these best-fit Hill coefficients values can be interpreted (Figure 14).
Figure 13. The effect of nickel on the electronic absorption spectrum of C198T HypB. Different shades represent different concentrations of nickel, between 0 μM Ni(II) (darkest), and 70 μM Ni(II) (lightest), added to a solution of 10 μM C198T HypB. (a) The full absorption spectra of C198T HypB show a LMCT at 320 nm that is caused by the N-terminal nickel-binding site. (b) Difference spectra obtained by subtracting the spectrum of the protein in the absence of added Ni(II) show the LMCT bands caused by nickel in the G-domain binding site, including a band at 340 nm.
Figure 14. Determining the nickel dissociation constant of nucleotide-free HypB proteins. Fractional saturation was determined by monitoring the 340 nm absorbance of 10 μM protein that was incubated with 0 – 100 μM Ni(II), in the absence of nucleotide. (a) In this representative experiment, C198T HypB bound nickel with a dissociation constant of 34 μM (Hill coefficient 2.6). (b) In this experiment, wild-type HypB bound nickel with an apparent dissociation constant of 4.3 μM (Hill coefficient 1.0).

It was important to determine the nickel $K_D$ in the presence of guanine nucleotides; however, these cause an intense band to arise in the electron absorption spectrum at 253 nm, which was found to interfere with the low intensity LMCT at 340 nm. For this reason, the nickel $K_D$ of HypB in the presence of GDP and GTP$_a$ was determined by competition against the metal-binding fluorescent probe mag fura 2 (MF2).

In the presence of GDP and GTP$_a$ C198T HypB binds nickel very well (Table 7). A previous study determined that wild-type HypB binds Ni(II) more tightly when it is loaded with a GTP analogue (GppCp) than when it is loaded with GDP [12]. This
experiment was repeated using the Gpp(NH)p GTP analogue, and wild-type HypB was found to bind nickel more tightly in the GTP\textsubscript{\alpha} state than the GDP state, although these data were not significant at the p = 0.05 level (p = 0.06; three replicates). In contrast, the apparent dissociation constant for C198T HypB is unaffected by the nucleotide-loaded state. HypB was assumed to bind nickel as (HypB)\textsubscript{2}Ni(II) instead of (HypB)Ni(II), based on size-exclusion data in 2.5 % glycerol (see \emph{C198T HypB quaternary structures} [pg 47]). If nickel-binding to HypB occurs in the (HypB)\textsubscript{2}Ni(II) complex, the same overall trend would occur, although the \(K_D\) of nickel-binding would be smaller.

<table>
<thead>
<tr>
<th></th>
<th>GDP state</th>
<th>GTP\textsubscript{\alpha} (Gpp(NH)p) state</th>
<th>No nucleotide\textsuperscript{†}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type HypB</td>
<td>(K_D = 3 \pm 1 \mu\text{M})</td>
<td>(K_D = 1.3 \pm 0.3 \mu\text{M})</td>
<td>(K_D = 4.3 \mu\text{M})</td>
</tr>
<tr>
<td>C198T HypB</td>
<td>(K_D = 14 \pm 3 \mu\text{M})</td>
<td>(K_D = 12 \pm 6 \mu\text{M})</td>
<td>(K_D = 40 \pm 10 \mu\text{M})</td>
</tr>
</tbody>
</table>

\textsuperscript{†} The no nucleotide data were not obtained through MF2 competition experiments, and were determined using electron absorption spectroscopy by following the LMCT band at 340 nm (Figure 13, 14).

\textbf{Table 7.} Nickel \(K_D\) for HypB in the presence of guanine nucleotides. The apparent dissociation constant of MF2 was experimentally determined to be \(2.0 \pm 0.7 \mu\text{M}\) for Ni(II) under the buffer conditions used, which is in agreement with previous work [12]. Wild-type HypB is known to bind nickel more strongly in the GTP\textsubscript{\alpha}-loaded state than the GDP-loaded state.

According to the current model, HypB binds nickel before it hydrolyzes GTP, and conversion of GTP to GDP/P\textsubscript{i} results in a weaker nickel affinity in the G-domain site, and also promotes formation of the HypB-HypA complex (Figure 2 [12,13]). Once HypA receives nickel, it no longer binds HypB, and this process, which depends on GTP/GDP cycling by HypB, forces unidirectional transfer of nickel from HypB to HypA. Based on
nickel affinity data (Table 7), the C198T mutation may have prevented the protein from adjusting nickel affinity based on the nucleotide state.

Two preliminary experiments show that C198T HypB, like wild-type HypB, can bind Zn(II) in the G-domain. First, Zn(II) was able to compete with Ni(II) for binding to C198T HypB, and the results indicate that zinc can bind with a dissociation constant of approximately 0.1 µM (Figure 15), which is similar to the reported Zn(II) dissociation constant of HypB in the absence of nucleotide (0.17 ± 0.14 µM [12]). C198T HypB continues to bind zinc with high affinity in the presence of nucleotide, and can compete with mag fura 2 for the metal. This experiment finds a low µM dissociation constant in the presence of guanine nucleotides GDP and GTPa (Figure 16), similar to the reported Zn(II) dissociation constant of wild-type HypB (0.1 µM for both GDP and GTPa [12]). Together these two experiments strongly suggest that C198T HypB can bind zinc, with low µM or high nM KD, similar to the wild-type protein. Zn(II) competition experiments need to be further explored to accurately determine the C198T HypB zinc dissociation constant and to determine the dissociation constant for the wild-type protein under these conditions.
Figure 15. Zinc-binding to the G-domain of C198T HypB. A solution containing 1 µM C198T HypB and 120 µM Ni(II) was incubated overnight with 0 – 8 µM Zn(II) at 4 °C under anaerobic atmosphere. When Zn(II) displaces Ni(II) it causes loss of the LMCT at 340 nm. The effect of Zn(II) on the fractional saturation of C198T HypB with Ni(II) was modeled by Dynafit, which indicated that Zn(II) bound to the G-domain site with $K_D \sim 0.1$ µM. This result demonstrates that after removing the C198 ligand, Zn(II) continues to bind to the G-domain with high affinity.
Figure 16. Zinc-binding to the G-domain of C198T HypB in the presence of nucleotides. (a) C198T HypB competes for zinc in the presence of 500 µM GDP (K_D ~0.02 µM; data points indicated by circles). There is a high degree of error in this data, and to illustrate this theoretical experiments where the K_D falls between 0.2 µM and 0.002 µM were modeled. Any curve that has a K_D between 0.2 µM and 0.002 µM would fall in the shaded area. (b) C198T HypB competes for zinc in the presence of GTP_a (K_D ~0.03 µM). If the K_D is between 0.3 µM and 0.003 µM the curve would be expected to be within the shaded area. It is qualitatively clear that C198T HypB binds zinc; however, the experiment should be repeated, using λ_ex = 370 nm (instead of λ_ex = 335 nm) to more accurately determine the apparent dissociation constant for zinc.

Electron absorption spectroscopy and fluorescent competition experiments show that C198T HypB binds both Ni(II) and Zn(II); however, it is unclear whether the mutant can selectively transfer Ni(II) to HypA. In the case of wild-type HypB, Ni(II) is transferred to HypA when HypB is in the GDP state, and Zn(II) is withheld in both the GDP and GTP_a states [12]. Determining whether the transfer of nickel from C198T HypB
to HypA is metal selective, and policed by the nucleotide state, might help to illuminate why C198T HypB is unable to mature [NiFe]-hydrogenase in *E. coli*.

**C198T HypB quaternary structures**

In a glycerol-free buffer (Buffer F, Table 3) 100 µM Ni(II) caused C198T HypB to form oligomers. If 100 µM GTP$_a$ was also present the protein mostly formed monomers and dimers (Figure 17). Guanine nucleotides are present in *E. coli* at millimolar concentrations [39], and it is therefore likely that C198T HypB forms monomers and dimers inside *E. coli* rather than higher-order oligomers. These results, however, indicate that mutating this metal-binding residue could have had dramatic consequences on the repertoire of quaternary structures formed by HypB. C198T HypB is also prone to aggregation at Ni(II) concentrations $\geq$ 130 µM (data not shown). To minimize oligomerization and precipitation of C198T HypB, 2.5 % (v/v) glycerol was used (Buffer D, Table 3); however, under these conditions neither 100 µM GDP on its own, nor 100 µM Ni(II) and 100 µM GDP together, caused C198T HypB to form dimers (Figure 18C). These experiments used protein from a single round of purification, however the wild-type protein was also predominantly monomeric in the presence of 100 µM GDP, with only a small dimer peak observed in the trace (Figure 18A). C198T HypB therefore behaves differently from wild-type HypB by forming higher-order complexes or, on the other hand, exclusively monomers depending on the buffer conditions. It is unclear which buffer condition (no glycerol vs. 2.5 % glycerol) better represents the periplasm of *E. coli*. It is also unclear whether dimer formation is important to [NiFe]-hydrogenase maturation at all; previous work on a mutant of HypB that cannot form homodimers found that this monomeric species could still mature [NiFe]-hydrogenase [26].
One protein-protein complex that is, according to the current model, essential to the maturation of [NiFe]-hydrogenase is HypB-HypA (Figure 2). In buffer with 2.5 % glycerol and 100 µM GDP, wild-type HypB forms a complex with HypA_{str} (Figure 18A). GDP was used in these analytical size-exclusion chromatography experiments, as GDP is known to promote the formation of the HypB-HypA_{str} complex [12]; however, C198T HypB was unable to form a complex with HypA_{str} under these conditions, even though wild-type HypB can do so (Figure 18A, 18B). It is therefore possible that the C198T mutation has prevented the formation of this critical complex. While it is surprising that a mutation to C198, a metal-binding site and Switch II motif residue, would cause dramatic structural changes at the level of quaternary structure, these size-exclusion chromatography results point towards this possibility. These experiments need to be repeated under different nucleotide conditions to more clearly decipher whether C198T HypB can form a complex with HypA_{str} and whether this complex disengages upon Ni(II) delivery. Furthermore, several wild-type HypB controls must be conducted, and the experiments must be replicated with separately purified C198T HypB.
Figure 17. Impact of nickel on the quaternary structures of C198T HypB and wild-type HypB in glycerol-free buffer. (a) Nickel causes an increase in the wild-type HypB dimer population. (b) In the case of C198T HypB, oligomers (dimers, tetramers, octamers) form in the presence of nickel. Guanine nucleotide (100 µM Gpp(NH)p), however, causes C198T HypB to form predominantly monomers and dimers. The absorbance values of each trace were normalized to make the total area under the curves equal.
Figure 18. Size exclusion chromatography of HypB proteins and HypA. All traces reflect experiments conducted in the presence of 2.5% glycerol and 100 µM GDP. (a) The dotted line shows GDP-HypB, which forms a small amount of dimer (~13 mL), however mostly forms monomer (~15 mL). The solid line is 1:1 incubation of WT HypB and HypAstr. A HypB-HypAstr complex is visible at ~14 mL. (b) The dotted line shows GDP-C198T HypB on its own, the solid line 1:1 incubation of C198T HypB and HypAstr. No protein complex was observed under these conditions. (c) The black line is GDP-C198T HypB on its own, the green line GDP-C198T HypB in the presence of 100 µM Ni(II). C198T HypB dimer was not observed under these conditions.
Conclusions and future work

Cells that express C198T HypB rather than wild-type HypB have a hydrogenase deficient phenotype, so the consequence of the mutation on the function of HypB is evidently severe. While the \textit{in vivo} effect is obvious, the etiology of hydrogenase deficiency, in terms of structural and functional defects, has been difficult to pinpoint. C198T HypB can be purified with appropriate molecular weight, adapts a secondary structure that resembles that of wild-type HypB, and carries out several of the essential activities of HypB. For instance, it binds Ni(II), although binding is weaker than the wild-type protein, and conducts GTP hydrolysis with an efficiency that resembles wild-type HypB. Furthermore, metal-dependent and metal selective inhibition of GTP hydrolysis continues to occur in C198T HypB, with activity resembling that of the wild-type protein. This result was particularly surprising, given that the purpose of the C198T mutation was to decouple the essential activities of metal-binding and GTP hydrolysis in HypB, and was based on experimental evidence from \textit{HpHypB} [16]. Also surprisingly, nickel only affected the \( K_M \) of GTP hydrolysis for both wild-type and C198T HypB, and \( k_{\text{cat}} \) was not affected by nickel binding. This contrasts with the effect of zinc on GTP hydrolysis, which results in a large reduction in \( k_{\text{cat}} \). Given that the concentration of GTP is high in \textit{E. coli} [39], it is possible that only zinc binding has a relevant effect on GTP hydrolysis \textit{in vivo}, a result that emphasizes a role for this GTPase in metal selectivity.

It is possible that communication between the GTPase and metal-binding sites was impacted in a way that was not initially predicted. For instance, MF2 competition experiments suggest that nucleotide-dependent nickel affinity, observed in wild-type \textit{E. coli} HypB [12], might not occur in C198T HypB. It may also be the case that complex
formation with HypA was somehow affected by this mutation to the metal site, given preliminary data from size-exclusion chromatography. This possibility needs to be further explored by further size-exclusion experiments, as well as orthogonal methods such as cross-linking. It is not known whether C198T HypB can selectively transfer nickel to HypA. Determining whether nickel transfer occurs would involve electron absorption spectroscopy to follow the loss of the LMCT in the C198T HypB G-domain, as well as mass spectrometry to observe transfer of a 58 Da component that corresponds to nickel.

It is also possible that the dramatic phenotype is not actually the result of a significant defect in function. For instance, it could be the case that nickel handoff amongst HypB, HypA, and unknown upstream components requires that the nickel $K_D$ for each factor be within a particular window. If this is the case, even the moderate increase in nickel $K_D$ caused by the C198T mutation might result in a failure to produce [NiFe]-hydrogenase. Furthermore, hydrogenase deficiency could be the consequence of many small impacts on the function of HypB, none of which would individually cause [NiFe]-hydrogenase deficiency, but all of which together prevent its maturation. For instance non-dramatic defects in protein-protein interactions, nucleotide regulation, and nickel affinity might together make [NiFe]-hydrogenase maturation impossible.

While the mutation is small, the in vivo consequences are striking. Learning why [NiFe]-hydrogenase maturation is inaccessible to this system could shed light on the role of HypB in the maturation of this enzyme and could be illuminating with respect to the role of the NTPase in nickel enzyme maturation.
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Figure A1. An ESI-MS spectrum (positive ion mode) of C198T HypB, after desalting. The major peak (31,431.38 Da) is very close to the expected mass of apo-C198T HypB (31,431.8 Da). The second most common mass observed by ESI-MS (+35 Da) corresponds to a Cl⁻ salt of C198T-HypB, the third most common (+22 Da) to the Na⁺ salt of C198T HypB, and the fourth most common (+58 Da) to the mass of Ni(II)-C198T HypB. The ESI-MS spectrum indicates that C198T HypB was successfully purified with the correct primary structure. ESI-MS can provide additional information on the oxidation state of proteins, which lose protons upon the formation of disulphide bonds.
Figure A2. An ESI-MS spectrum (positive ion mode) of wild-type HypB, after desalting. The major peak (31,433.35 Da) is close to the expected mass of apo-WT HypB (31,433.8 Da). The second most common mass observed by ESI-MS (+ 21 Da) approximately corresponds to the Na\(^+\) salt of WT HypB, the third most common (+ 38 Da) corresponds to the K\(^+\) salt of WT HypB, and the fourth most common (+ 60 Da) approximately corresponds to the mass of Ni(II)-WT HypB.
Figure A3. Impact of nickel on the secondary structure of wild-type HypB and C198T HypB. As nickel increases from 0 µM Ni(II) to 100 µM Ni(II), the band at ~205 nm increases in intensity, and the band at ~220 nm becomes less intense for both (a) C198T HypB and (b) wild-type HypB. C198T HypB is affected by nickel in the same fashion as wild-type HypB. These samples were prepared under anaerobic atmosphere by diluting the purified protein 1:10 into water. After dilution, the buffer contained 2.5 mM Hepes (pH 7.5), 20 mM NaCl, and 100 µM TCEP. This experiment should be repeated in a more strongly buffered, CD appropriate solution. Potassium phosphate buffer was not used due to the low solubility of nickel-phosphate [33], and Hepes at 25 mM was not used, as it absorbs strongly at and below 200 nm [32].
Impact of glycerol on the GTPase activity of wild-type HypB. In this experiment, 4 µM wild-type HypB was incubated with 280 µM GTP for 20 min at 37 °C, in Buffer F (Table 3) with different percent concentrations of glycerol (three technical replicates). At 5 % glycerol, the rate of GTP turnover was impacted, and at 10 % glycerol the turnover of GTP by wild-type HypB was entirely inhibited. It is likely that this effect is not related to quaternary structure, as dimer formation does not impact GTP hydrolysis by HypB [26]. Protein-free, and buffer-only controls were only prepared for the 0 %, 0.05 %, 1.25 %, 5 % and 10 % glycerol conditions (three technical replicates).
[task]
   data = equilibria
   task = fit

[mechanism]
   M + MF2 <=> M.MF2 : Kd1 dissoc.
   M + HypB <=> M.HypB : Kd2 dissoc.

[concentrations]
   MF2 = 1
   HypB = 5

[constants]
   Kd1 = 2.0
   Kd2 = 1 ?

[responses]
   M.MF2 = 1
   MF2 = 0

[equil]
   variable M
   offset auto ?
   file
   /Users/Conor/C198T_HypB/Input.txt

[output]
   directory /Users/Conor/C198T_HypB/Output

[end]

**Figure A5.** DynaFit script for competitive binding of nickel by HypB and mag fura 2.
Figure A6. DynaFit script for competitive binding between nickel and zinc for a single site in HypB.