

Escherichia coli SlyD, More Than a Ni(II) Reservoir

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S Supporting Information

ABSTRACT: SlyD interacts with HypB and contributes to nickel insertion during [NiFe]-hydrogenase biogenesis. Herein, we provide evidence of SlyD acting as a nickel storage determinant in *Escherichia coli* and show that this Ni(II) can be mobilized to HypB in vitro even under competitive conditions. Furthermore, SlyD enhances the GTPase activity of HypB, and acceleration of release of Ni(II) from HypB is more pronounced when HypB is GDP-bound. The data support a model in which a HypB–SlyD complex establishes communication between GTP hydrolysis and nickel delivery and provide insight into the role of the HypB–SlyD complex during [NiFe]-hydrogenase biosynthesis.

Central to microbial energy metabolism as well as fuel cell applications, [NiFe]-hydrogenases catalyze the oxidation of hydrogen gas and/or the reverse reaction.¹ This challenging chemistry occurs at an intricate organometallic center that is assembled by several accessory proteins.^{1,2} In *Escherichia coli*, the HypCDEF proteins are responsible for the synthesis of diatomic ligands and delivery of the decorated iron center to the hydrogenase precursor protein.¹ The subsequent nickel insertion is conducted by HypA, HypB, and SlyD.^{1,2}

While HypA is anticipated to serve as a scaffold for the nickel insertion complex during enzyme maturation,^{2,3} HypB catalyzes the GTP hydrolysis that is essential for hydrogenase maturation.^{4–6} *E. coli* HypB also has two metal-binding sites (Figure S1 of the Supporting Information), one that includes the CXXCXC sequence at the N-terminus of the protein and binds nickel with picomolar affinity [thus termed the high-affinity site (HAS)] and a second in the GTPase domain (G-domain) that binds nickel or zinc with low micromolar affinity [low-affinity site (LAS)].⁷ SlyD is a multidomain protein harboring both protein folding and metal binding activities.^{8–10} The unique C-terminal domain of SlyD (Figure S1) features a high concentration of potential metal-binding residues and chelates multiple nickel ions in vitro.⁹ Mutation of the residues contributing to either of the metal sites of HypB or deletion of the metal-binding domain (MBD) of SlyD compromises hydrogenase maturation.^{11,12}

E. coli HypB lacks the polyhistidine stretch that allows some HypB homologues to bind nickel with high capacity but low affinity.^{2,13} Consequently, nickel storage in this organism is a proposed role of SlyD.^{8,14} To test this hypothesis, we compared the Ni(II) content and hydrogenase activity of a wild-type strain with those of a Δ slyD strain. Cell cultures were first exposed to Ni(II) under anaerobic conditions to allow the *E. coli* to accumulate Ni(II) and potentially store these metal ions

in SlyD. The bacteria were then washed and transferred to untreated TYEP medium or TYEP medium treated with dimethylglyoxime (DMG), and activity was assessed after an additional growth step. DMG treatment is expected to mimic nickel limiting conditions because this chelator selectively binds nickel with high affinity.^{15,16} Under these conditions, the cytoplasmic nickel content and the hydrogenase activity of the Δ slyD strain decreased to 8 and 11% of those of the wild-type strain, respectively, an effect that is much more dramatic than when the bacteria were cultured without DMG (Tables S1 and S2 of the Supporting Information). These results afford physiological evidence that SlyD is a Ni(II) reservoir in *E. coli* and that bacteria without SlyD are highly dependent on exogenous nickel for [NiFe]-hydrogenase assembly.

Given the observation that SlyD could store Ni(II) ions and that it forms a complex with HypB,^{12,14} we postulated that this protein could function as a source of Ni(II) for metallating HypB. To test this model, we mixed SlyD bound to 1 equiv of Ni(II) with apo HypB and assessed the transfer of metal from SlyD to HypB. We used electrospray ionization mass spectrometry (ESI-MS) for analysis, which allowed simultaneous detection of the metalation state of both proteins (Figure S2 of the Supporting Information). Incubating holo SlyD with apo HypB led to accumulation of Ni(II)-bound HypB with a concomitant decrease in the amount of holo SlyD (Figure 1A and Figure S2). Although HypB has two metal-binding sites,⁷ only a single Ni(II) per HypB monomer is detected in these spectra. The fact that SlyD populates only the HAS of HypB was confirmed by the lack of transfer of metal from holo SlyD to the HypB mutant C2,S,7A (data not shown), which has a disrupted HAS but an intact LAS.⁷

To determine if heterodimer formation is necessary for metal transfer, we dialyzed SlyD bound to 1 equiv of metal against an apo HypB sample. The rate of transfer of nickel to HypB was considerably reduced compared to that achieved when the two proteins were incubated together (Figure 1A). To assess whether metal transfer could occur under more competitive conditions, transfer of Ni(II) from SlyD to HypB was monitored in the presence of two metal chelators, EGTA and glycine. EGTA forms a 1:1 complex with Ni(II) with an apparent K_D of $\sim 10^{-11}$ M at pH 7.5, whereas glycine coordinates Ni(II) with a weaker affinity in the micromolar range.¹⁷ While the transfer of nickel from SlyD to HypB remained unchanged in the presence of 1 mM glycine (data not shown), inclusion of EGTA led to a decrease in the Ni(II) transfer rate compared to that in the absence of a competitor

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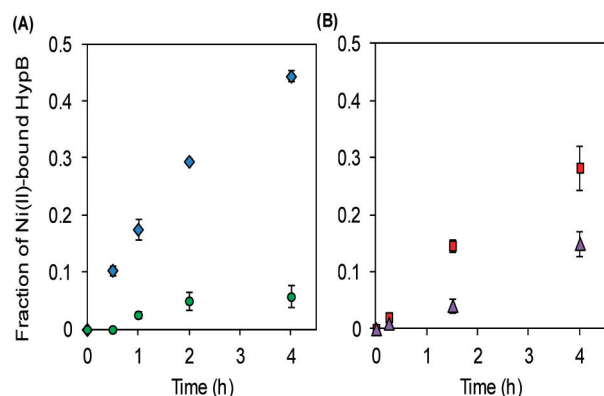


Figure 1. Transfer of Ni(II) from SlyD to HypB monitored by ESI-MS. (A) Incubating SlyD (100 μ M) bound to 1 equiv of Ni(II) with apo HypB (100 μ M) leads to an increase in the amount of Ni(II)-bound HypB (diamonds). Slower metal transfer was observed if the proteins were separated by a membrane (circles). See the Supporting Information for a discussion of the protein concentrations. (B) Ni(II)-bound SlyD (100 μ M) was incubated with apo HypB (100 μ M) in the presence of 1 mM EGTA (squares). Less holo HypB was obtained when 100 μ M Ni(II) was added to apo HypB (100 μ M) in the presence of 1 mM EGTA (triangles) without SlyD. Each data point is an average from at least three independent experiments, and the error bars represent one standard deviation.

(Figure 1 and Figure 2S). However, HypB metalation was faster with the Ni(II)–SlyD complex than if Ni(II) was provided as a [Ni–EGTA]²⁻ complex (Figure 1B). Together, these data indicate that Ni(II) can relocate from SlyD to the HAS of HypB even under competitive circumstances and heterodimer formation is a prerequisite for the achievement of favorable conditions.

The intrinsic GTP hydrolysis of *E. coli* HypB is very slow ($k_{\text{cat}} \approx 0.2 \text{ min}^{-1}$).^{6,18} NTPases can utilize protein–protein interactions to accelerate low hydrolysis rates,^{19,20} so we tested whether SlyD could enhance the HypB GTPase activity. While HypB with Ni(II) bound at the HAS (holo HypB) hydrolyzed GTP with a k_{cat} of $0.27 \pm 0.06 \text{ min}^{-1}$, the addition of SlyD resulted in a 3-fold increase in the catalytic rate (Table 1). SlyD is the first factor observed to positively contribute to the enzyme activity of HypB. The MBD of SlyD is necessary for this process because the same effect was not observed with SlyD_{1–146} (Table 1). The k_{cat} of HypB mutant C2,5,7A is analogous to that of wild-type HypB, indicating that the HAS does not influence the GTPase activity of the protein (Table 1). In agreement with this conclusion, SlyD produces an increase in the GTPase activity of C2,5,7A HypB comparable to that of wild-type HypB (Table 1).

To assess the influence of SlyD on GTP hydrolysis when HypB has metal loaded in both metal sites, holo HypB with either Ni(II) or Zn(II) bound to the LAS was examined. The

GTPase activity was reduced when the LAS was metalated, as expected (Table 1).¹⁸ Inclusion of SlyD in the reaction mixture reversed the metal-dependent inhibition, leading to an ~ 10 -fold increase in the hydrolysis rate when HypB containing Zn(II) at the LAS was used (Table 1). In contrast, SlyD_{1–146} could not rescue the compromised GTP hydrolysis activity of HypB with metal loaded in the LAS. Given that wild-type SlyD can bind multiple Ni(II) or Zn(II) ions while SlyD_{1–146} is unable to do so (ref 9 and unpublished data), we postulated that metal-dependent inhibition of HypB enzymatic activity is reversed because of competitive metal chelation of the LAS metal of HypB by the MBD of SlyD. In agreement with this model, ESI-MS revealed that the transfer of metal from the LAS of HypB to SlyD was $\sim 95\%$ complete within 15 min (Figure S3 of the Supporting Information) and remained unaffected when HypB was bound to GDP or GMP-PNP, or if SlyD was preloaded with 1 equiv of Ni(II) (data not shown). SlyD_{1–146} does not lead to any detectable loss of metal from HypB (Figure S4 of the Supporting Information).

Previous work demonstrated that heterodimer formation with SlyD increases the rate of release of metal from the N-terminal HAS of HypB to a colorimetric chelator.¹² To establish features of SlyD that are necessary to modulate the HAS of HypB, several apo SlyD variants were incubated with holo HypB in the presence of EGTA, followed by ESI-MS analysis. Very little apo HypB was detected when it was mixed with EGTA alone, but the addition of wild-type SlyD or the triple mutant [that has reduced capacity and affinity for Ni(II) because of the removal of all three pairs of Cys residues⁹] led to similar acceleration of the release of Ni(II) from HypB (Figure S5 of the Supporting Information). The application of ESI-MS revealed that Ni(II)-bound SlyD was not detected at any time point, indicating that the nickel released from HypB was not transferred to SlyD. As expected,¹² SlyD_{1–146} (lacks MBD), Δ flap SlyD (deletion of a loop in the IF domain abrogates interaction with HypB¹²), and SlyD-MBD (contains only residues 147–196) had no effect on holo HypB (Figure S5), confirming that complex formation and the MBD are both necessary to accelerate the release of Ni(II) from HypB.

To assess whether nucleotide could modulate the high-affinity Ni(II) site of HypB, release of metal from HypB to EDTA was monitored in the presence of GDP or the GTP analogue GMP-PNP using electronic absorption spectroscopy. Coordination of Ni(II) to the Cys ligands of the HAS of HypB results in a ligand to metal charge transfer band at 320 nm ($\epsilon_{320} = 7300 \text{ M}^{-1} \text{ cm}^{-1}$).⁷ Given the observation that the nickel from holo HypB is not transferred to SlyD (discussed above), the decrease in the magnitude of the signal at 320 nm should correlate with the release of Ni(II) from HypB into the solution containing EDTA ($K_{\text{D}} = 2.7 \times 10^{-16} \text{ M}$ at pH 7.5).¹⁷ Addition of apo SlyD stimulates the release of nickel from the HypB–GMP-PNP complex to the same extent as from HypB in the

Table 1. Rates of GTP Hydrolysis of HypB under Saturating Conditions^a

metal bound at the G-domain	HypB _{WT}	HypB _{WT} with SlyD _{WT}	HypB _{WT} with SlyD _{1–146}	HypB _{C2,5,7A}	HypB _{C2,5,7A} with SlyD _{WT}
–	0.27 \pm 0.06	0.82 \pm 0.04	0.36 \pm 0.08	0.21 \pm 0.05	0.75 \pm 0.08
Ni(II)	0.13 \pm 0.03	0.87 \pm 0.01	0.12 \pm 0.03	0.13 \pm 0.02	0.78 \pm 0.09
Zn(II)	0.07 \pm 0.04	0.91 \pm 0.09	0.06 \pm 0.04	0.06 \pm 0.02	0.88 \pm 0.05

^aThe values reported are k_{cat} values (min^{-1}). Assays were performed using 1 μ M holo HypB with nickel in the HAS, 450 μ M GTP, and, when required, SlyD at a final concentration of 50 μ M. The k_{cat} values are averages and standard deviations from more than three independent experiments. To load the G-domain metal site, HypB was incubated with a 10-fold molar excess of Ni(II) or a 1.5-fold molar excess of Zn(II).

absence of nucleotide, whereas metal release is further enhanced when SlyD is mixed with the HypB–GDP complex (Figure 2). Fits of the metal release profiles to a first-order

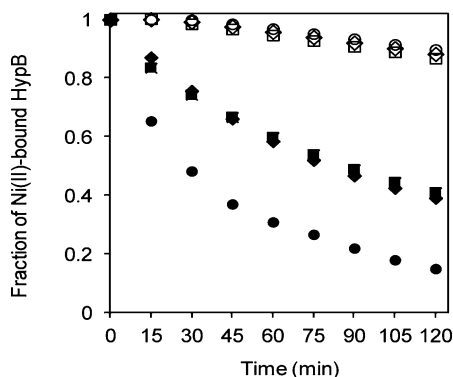


Figure 2. Release of metal from HypB to EDTA monitored by electronic absorption spectroscopy. The decrease in the fraction of 100 μM Ni(II)-bound HypB in the presence of 1 mM EDTA (empty symbols) was monitored at 320 nm. Release of metal from HypB on its own (\square) is not affected upon addition of GDP (\circ) or the GTP analogue (\diamond). Including 100 μM SlyD (filled symbols) results in an increase in the rate of metal release. Release of metal from HypB bound to the GTP analogue is similar (\blacklozenge), whereas SlyD further accelerates the release of metal from the HypB–GDP complex (\bullet).

decay event yield $t_{1/2}$ values of ~ 24 h and 73 min for holo HypB in the absence and presence of SlyD, respectively. The half-life for the GMP-PNP-loaded HypB incubated with SlyD is unaffected ($t_{1/2} \sim 75$ min), whereas that of the GDP-bound form decreases to ~ 33 min. Analogous nucleotide-dependent enhancement in interactions has been observed between MeaB²⁰ and UreG¹⁹ GTPases and their partner proteins.

GTP hydrolysis is mandatory for maturation of the hydrogenase enzyme,⁶ but it is not clear how energy derived from this reaction is utilized for assembling the [NiFe] center. The results presented here provide evidence that GTP hydrolysis can regulate the loss of Ni(II) from the HAS of HypB. The enhanced release of metal from the holo HypB–SlyD heterodimer upon GDP binding is consistent with a model in which GTP hydrolysis precedes delivery of Ni(II) to the precursor enzyme (Figure S6 of the Supporting Information) and may be due to a more favorable interaction between SlyD and GDP-bound HypB. Insertion of nickel into the hydrogenase precursor appears to be gated by GTP hydrolysis, and this link between hydrolysis and metal release is achieved through SlyD.

In summary, SlyD functions as a reservoir of Ni(II) for hydrogenase biosynthesis under nickel-limiting conditions, but its role extends to optimal biogenesis of the [NiFe]-hydrogenase by modulating the activities of HypB (Figure S6). These discoveries provide insight into how protein–protein interactions as well as the GTPase function of HypB can be exploited to ensure the timely delivery of the metal ion. Furthermore, the effects mediated by protein–protein contacts highlight how the coordinated actions of multiple protein factors can overcome the barriers for efficiently inserting the nickel ion into a partially assembled hydrogenase active site.

■ ASSOCIATED CONTENT

■ Supporting Information

Detailed experimental procedures, tables with nickel content and hydrogenase activity, representative reconstructed mass spectra, metal release profiles for SlyD variants, proposed model for insertion of Ni(II) into the hydrogenase precursor. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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