The role of complex formation between the Escherichia coli hydrogenase accessory factors HypB and SlyD*

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The Escherichia coli protein SlyD is a member of the FK-506-binding protein family of peptidylprolyl isomerases. In addition to its peptidylprolyl isomerase domain, SlyD is composed of a molecular chaperone domain and a C-terminal tail rich in potential metal-binding residues. SlyD interacts with the [NiFe]-hydrogenase accessory protein HypB and contributes to nickel insertion during biosynthesis of the hydrogenase metalocenter. This study examines the HypB-SlyD complex and its significance in hydrogenase activation. Protein variants were prepared to delineate the interface between HypB and SlyD. Complex formation requires the HypB linker region located between the high affinity N-terminal Ni(II) site and the GTPase domain of the protein. In the case of SlyD, the deletion of a short loop in the chaperone domain abrogates the interaction with HypB. Mutations in either protein that disrupt complex formation in vitro also result in deficient hydrogenase production in vivo, indicating that the contact between HypB and SlyD is important for hydrogenase maturation. Surprisingly, SlyD stimulates release of nickel from the high affinity Ni(II)-binding site of HypB, an activity that is also disrupted by mutations that affect complex formation. Furthermore, a SlyD truncation lacking the C-terminal metal-binding tail still interacts with HypB but is deficient in stimulating metal release and is not functional in vivo. These results suggest that SlyD could activate metal release from HypB during metallation of the [NiFe] hydrogenase.

The assembly of the [NiFe] metalocenter of Escherichia coli hydrogenase 3 requires the participation of proteins encoded by the hyp (hydrogenase pleiotropy) genes hypAB-CDEF (reviewed in Refs. 1–3). HypA and HypC are replaced by the homologous HybF and HybG proteins, respectively, for the assembly of hydrogenases 1 and 2 (1, 2). HypC, HypD, HypE, and HypF participate in the biosynthesis of the Fe(CN)$_2$(CO) cluster and delivery to the hydrogenase precursor protein (4–6). The subsequent incorporation of nickel (7, 8) requires the GTPase HypB and HypA. These proteins were initially implicated in the nickel insertion step by genetic studies in which the hydrogenase deficiency resulting from chromosomal mutations was at least partially restored by growing the bacteria in excess nickel (9–13). E. coli HypB binds one nickel ion with a $K_d$ value in the picomolar range to the cysteines in the N-terminal CXXCG motif (referred to as the “high affinity site,” see Fig. 1 for domain architecture) (14). In addition, both HypB and HypA bind a nickel ion with micromolar affinity (14–17) as follows: HypA at a site that includes the conserved second residue His-2 (15, 16), and HypB to several conserved amino acids in the GTPase domain (referred to as the “low affinity site”) (14, 18). Whether one or a combination of these metal sites serve as a source of nickel for the hydrogenase enzyme has not yet been determined.

Upon searching for additional hydrogenase biosynthetic factors in E. coli, a protein called SlyD was identified in a complex with HypB and shown to play a role in hydrogenase production (19). SlyD is a member of the FK-506-binding protein (FKBP) family of peptidylprolyl isomerases (PPIases) (20, 21). In general, PPIases are capable of assisting in various protein folding processes by catalyzing the cis-trans-isomerization of a prolyl amide bond, but in many cases it is not clear that this activity is required for the physiological function of the protein (22, 23). SlyD, like many FKBP PPIases, including the E. coli ribosome-associated trigger factor (24) and periplasmic FkpA (25), also exhibits general molecular chaperone activity (26). SlyD shares with a subset of FKBPs an additional domain, termed IF (insert in the flap), which has been structurally characterized for an archaeal FKBP, MtFKBP17 from Methanococcus thermolithothrophicus, and may allow these proteins to bind unfolded or extended polypeptide chains (27, 28). Although they possess many attributes suitable for a housekeeping chaperone function, some PPIases bind only to a limited set of client proteins and appear to play a regulatory role in specific biochemical pathways (22, 23).

One unusual feature of SlyD is that it has a C-terminal 50-residue metal-binding domain. This domain is rich in the metal-binding amino acids, containing 15 histidines, 6 cysteines, and 7 aspartate/glutamate residues (see Fig. 1 for domain architecture).

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3 The abbreviations used are: FKBP, FK506-binding protein; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); EDC, (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride; PAR, 4-(2-pyridylazo)resorcinol; PPIase, peptidyl-prolyl isomerase; SlyD(3flap), SlyD with residues 107–111 deleted; TCEP, Tris(2-carboxyethyl)phosphine; ESI-MS, electrospray ionization-mass spectrometry; MBP, maltose-binding protein; CS, citrate synthase.
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...and is required for metal-dependent inhibition of the PPIase activity (29). An E. coli strain with a deletion of the slyD gene exhibits reduced nickel accumulation as well as low hydrogenase activity that can be fully restored by the addition of excess nickel to the growth media (19), suggesting that SlyD contributes to the insertion of nickel into apohydrogenase. Unlike many other bacterial HypB proteins, E. coli HypB lacks a polyhistidine stretch that can bind multiple nickel ions and serve in nickel storage (30–33). Given that SlyD can bind multiple metal ions (20, 29), it is possible that SlyD has assumed this storage function (19).

To investigate the role of SlyD in the metallation of apohydrogenase, we examined the SlyD-HypB complex in more detail. The site of interaction was localized in both proteins, and mutants of either HypB or SlyD that fail to form the complex in vitro are defective in activating hydrogenase in vivo. Furthermore, when SlyD binds to HypB it activates metal release from the N-terminal high affinity nickel-binding site. A SlyD variant lacking the C-terminal tail is capable of interacting with HypB but does not contribute to hydrogenase biosynthesis in vivo or stimulate metal release from HypB in vitro. These data support a role beyond nickel storage for SlyD in triggering metal release from HypB and promoting nickel insertion into the hydrogenase precursor protein.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction endonucleases, T4 DNA ligase and calf intestine phosphatase were obtained from New England Biolabs. *Pfu* DNA polymerase was purchased from Stratagene. Chromatography media were from GE Healthcare. Isopropyl β-D-thiogalactoside, Tris-(2-carboxyethyl)phosphine (TCEP), ampicillin, and kanamycin were purchased from BioShop (Toronto). All other reagents were analytical grade from Sigma. Primers (Table 1) were purchased from Sigma Genosys. Solutions were prepared with Milli-Q water, 18.2 megohms-cm resistance (Millipore), and the pH values of the buffers were adjusted with HCl or NaOH.

**HypB and SlyD Expression Vectors and Mutants**—The generation of HypB-pET, G domain-pET, and SlyD-pET in the pET24b vector (Novagen) and pBAD-SlyD in the pBAD24 vector (American Type Culture Collection, ATCC) was described previously (14, 19). To generate pET-SlyD-(1–146), a fragment of slyD coding for residues 1–146 was amplified from DH5α *E. coli* by using the D(1–146) primer set (Table 1). After purification with the QIAquick PCR purification kit (Qiagen), the PCR product was digested with the restriction enzymes NdeI and XhoI. The digested PCR product was isolated from agarose gel pieces by using a QIAquick kit (Qiagen) and ligated into the pET24b vector digested with NdeI and XhoI and ligated into pET24b vector digested with NdeI and XhoI and dephosphorylated with calf intestine phosphatase. Ligation products were transformed into XL-2 Blue *E. coli* (Stratagene). For *in vivo* experiments HypB was amplified from pET24b-HypB using the BpBAD primer set, and a fragment of slyD coding for residues 1–146 was amplified from DH5α *E. coli* using

![Figure 1](image-url)

**FIGURE 1. Domain organization of *E. coli* HypB and SlyD.** A. *E. coli* HypB consists of an NH2-CXXCXC motif involved in high affinity Ni(II) binding (14), a linker region, and a GTPase domain with a lower affinity metal-binding site. HypB proteins from some bacteria include a polyhistidine sequence in the linker, but *E. coli* HypB has only a total of four histidine residues in this region. A proline-containing sequence in the linker of HypB lacks a polyhistidine stretch that can bind multiple nickel ions and serve in nickel storage (30–33). Given that SlyD can bind multiple metal ions (20, 29), it is possible that SlyD has assumed this storage function (19).

**TABLE 1**

| PCR primers used for cloning and mutagenesis | Sequence |
|---------------------------------------------|----------|
| B11d76_forward                             | 5′-CTGCCAGGAGGCAAAC/ATGCTGAAAGTAAAAATG-3′ |
| B11d76_reverse                             | 5′-CGCTCAGCAGCAGT/GGCCTGCCGGCAACC-3′     |
| B19d76_forward                             | 5′-CGACGCTGAAGT/ACCTGAGAAGTTGAAAG-3′     |
| B19d76_reverse                             | 5′-CGACGCTGAAGT/ACCTGAGAAGTTGAAAG-3′     |
| B26d58_forward                             | 5′-CGACGCTGAAGT/ACCTGAGAAGTTGAAAG-3′     |
| B26d58_reverse                             | 5′-AATCATCGCTGGCT/CTGCAGTCTGGCTCGG-3′    |
| BPP29,32SS_forward                         | 5′-CAGGATCCATCTGGGACAAG/AAACCCCTG-CCG-3′ |
| BPP29,32SS_reverse                         | 5′-CAGGATCCATCTGGGACAAG/AAACCCCTG-CCG-3′ |
| D(1–146)-forward                           | 5′-AATCATCGCTGGCT/CTGCAGTCTGGCTCGG-3′    |
| D(1–146)-reverse                           | 5′-AATCATCGCTGGCT/CTGCAGTCTGGCTCGG-3′    |
| D(140–190)-forward                         | 5′-AATCATCGCTGGCT/CTGCAGTCTGGCTCGG-3′    |
| D(140–190)-reverse                         | 5′-AATCATCGCTGGCT/CTGCAGTCTGGCTCGG-3′    |
| D(jap)-forward                             | 5′-AATCATCGCTGGCT/CTGCAGTCTGGCTCGG-3′    |
| D(jap)-reverse                             | 5′-AATCATCGCTGGCT/CTGCAGTCTGGCTCGG-3′    |
| BpBAD_FORWARD                              | 5′-TTTTCTCAGTGCGAG/GTGCGATCGTCTGGCTCGG-3′ |
| BpBAD_reverse                              | 5′-TTTTCTCAGTGCGAG/GTGCGATCGTCTGGCTCGG-3′ |
| D(1–146)-pBAD_FORWARD                      | 5′-TTTTCTCAGTGCGAG/GTGCGATCGTCTGGCTCGG-3′ |
| D(1–146)-pBAD_reverse                      | 5′-TTTTCTCAGTGCGAG/GTGCGATCGTCTGGCTCGG-3′ |
the D(1–146) pBAD primer set. PCR products were treated as described above, except they were digested with the restriction enzymes NheI and XbaI and ligated into the pBAD24 vector digested with the same enzymes, to generate pBAD-HypB and pBAD-SlyD(1–146), respectively. To generate a construct coding for a fusion between the maltose-binding protein (MBP) and the C-terminal tail of SlyD, a fragment of slyD coding for residues 140–196 was amplified with the primer set D(140–196). The purified PCR product was treated as described above, digested with Ndel and XhoI, and ligated into the vector pBADL16 ((34) generously provided by Prof. C. T. Walsh, Harvard Medical School) digested with the same restriction enzymes and treated with calf intestine phosphatase. Plasmids coding for amino acid variants and deletion variants were generated by QuikChange PCR mutagenesis (Stratagene) using Pfu polymerase and pET-HypB, pBAD-HypB, pET-SlyD, and pBAD-SlyD as templates. The primers used to prepare HypB(P29S,P32S), HypB(28d36), HypB(11d76), HypB(19d76), SlyD, and SlyD-(1–146) pBAD primer set. PCR products were treated as described previously (14, 37). Parent plasmids were digested with DpnI before transforming the reaction mix into competent cells. For routine handling, plasmids were transformed into XL-2 Blue E. coli prepared as described for preparations of MBP-SlyD and MBP-SlyD variants were expressed in a BL21(DE3) strain of E. coli prepared as described for preparations of D(slyD) BL21(DE3) E. coli cells grown in media containing 1 mM Ni(II) (14). The proteins were purified by sequential DEAE, HiTrapQ, and Superdex S-200 columns, as described previously (14), and stored at ~80 °C in the Superdex buffer (25 mM Hepes, pH 7.6, 200 mM NaCl, 1 mM TCEP). To determine the oligomeric state of HypB, the purified protein was run on an S-200 gel filtration column calibrated with β-amylase (200 kDa), bovine serum albumin (66.5 kDa), chicken ovalbumin (45 kDa), and carbonic anhydrase (29 kDa) as standards. HypB (31.6 kDa) elutes at a volume consistent with a molecular mass of 65 kDa, demonstrating that it is a dimer under these conditions, in contrast to our previously reported experiments using an S-75 gel filtration column calibrated with β-amylase (200 kDa), bovine serum albumin (66.5 kDa), chicken ovalbumin (45 kDa), and carbonic anhydrase (29 kDa) as standards. HypB concentrations were estimated using their ε280 values as predicted by the ExPaSy ProtParam program for the fully reduced proteins (36). Protein molecular weights were determined by ESI-MS and compared with the predicted values. The data were consistent with the loss of the N-terminal Met for all HypB variants except the GTPase domain as follows: HypB(P29S,P32S) 31,412.0 Da (predicted 31,413.7 Da), HypB(28d36) 30,554.0 Da (predicted 30,554.8 Da), HypB(19d76) 25,186.0 Da (predicted 25,186.8), and HypB(11d76) 24,250.0 Da (predicted 24,252.9 Da.). ESI-MS data for HypB and the GTPase domain were as reported (14). Data for SlyD and its variants were consistent with retention of the N-terminal methionine as follows: SlyD 20,853.0 Da (predicted 20,852.8 Da), SlyD-(1–146) 15,833.2 Da (predicted 15,833.6 Da), and SlyD(Δflap) 20,338.0 Da (predicted 20,339.2 Da). The observed molecular weight of the MBP-SlyD(140–196) fusion protein was consistent with removal of the N-terminal Met, 48,796.0 Da (predicted 48,797.4 Da).

Iodoacetamide Modification of SlyD—Purified SlyD (1 ml of 500 μM) was treated with 2 mM TCEP for 20 min and then with 50 mM iodoacetamide for 30 min with stirring at room temperature. The sample was then dialyzed against two changes of buffer (3 liters of 10 mM Hepes, 100 mM NaCl, pH 7.6) to remove the modification reagent and analyzed by ESI-MS before use. Modified SlyD has a mass of 21,196.0 Da consistent with all six cysteines having a carbamidomethyl modification (predicted 21,195.2 Da).

Circular Dichroism—CD spectra were recorded on a Jasco J-710 spectropolarimeter with an optical path length of 1 mm. The protein concentrations were 10–30 μM in 100 mM potassium phosphate buffer, pH 7.0.

GTPase Activity—To assay GTPase activity, released phosphate was detected by using Malachite Green in the presence of ammonium molybdate based on an adaptation of a published method as described previously (14, 37).

PPase Activity—A protease-free assay was used to measure the PPase activity of SlyD and the variants (38). The substrate, succinyl-αl-αl-Pro-Phe-nitroanilide (Bachem Bioscience), was dissolved in trifluoroethanol that had been dried over sieves and 0.47 M LiCl. The reactions contained 35 mM Hepes, pH 7.6, and 1 μM protein and were incubated at 10 °C prior to the addition of 71 μM substrate. Isomerization was monitored at 330 nm on a Cintra 40 spectrophotometer and fit to a single exponential decay. The uncatalyzed rate was also measured, and the second-order rate constant was calculated as follows: kcat/Km = (kobs − kuncat)/[enzyme].

Metal Binding and EGTA Competition—For all HypB variants we initially investigated whether the purified proteins had an electronic absorption band at 320 nm, which is diagnostic for bound nickel, and then prepared apoprotein from each of these proteins to test whether they bind stoichiometric nickel with an affinity comparable with wild-type HypB (Table 2) (14). Purified HypB variants were incubated with 20 mM EDTA and 2 mM TCEP in an anaerobic glove box for 72 h. The proteins were gel filtered twice through PD-10 columns equilibrated with 25 mM Hepes, pH 7.5, 100 mM NaCl. To test their reduction state, all proteins were treated with DTNB after dilution into 6 M guanidinium hydrochloride, and the absorbance at 412 nm was measured. Isomerization was monitored at 300 nm on a J-710 spectropolarimeter with an optical path length of 1 mm.

Metal Release Assay—HypB, as purified, was dialyzed to a final protein concentration of 5 μM into 25 mM Hepes, pH 7.5, 200
TABLE 2

| Name                  | $e_{380}$ value* | $K_d$       |
|-----------------------|-----------------|-------------|
|                        | $M$            | $M$         |
| HypB                  | (7.3 ± 0.1) $\times 10^3$ | (1.3 ± 0.2) $\times 10^{-13}$ |
| HypB(P29S,P32S)       | (7.5 ± 0.3) $\times 10^3$ | (1.2 ± 0.2) $\times 10^{-13}$ |
| HypB(28d36)           | (7.4 ± 0.2) $\times 10^3$ | (1.0 ± 0.4) $\times 10^{-13}$ |
| HypB(19d76)           | (8.5 ± 0.5) $\times 10^3$ | (1.9 ± 0.3) $\times 10^{-13}$ |
| HypB(11d76)           | (10.2 ± 0.3) $\times 10^3$ | (1.8 ± 0.4) $\times 10^{-13}$ |

* Data were measured by using direct titration with Ni(II). In some cases metal analysis of Ni(II)-HypB was also performed on protein incubated with nickel followed by gel filtration, and the extinction coefficients at 320 nm were within 5% of the listed values.

mm NaCl (buffer A) containing 100 μM PAR. The release of metal was monitored every 5 min by an increase in the absorbance at 500 nm due to the formation of the metal-PAR complex (39). To determine total metal in the sample ($A_{\text{max}}$), an aliquot of the same sample was treated with 100 μM parahydroxymercuribenzoic acid, and the data were converted to metal bound (100 × (1 − $(A_{\text{max}})$)) at a given time point and then fit to a single exponential decay equation. Data of $k_{\text{off}}$ versus SlyD were fit to a saturation equation to estimate the maximal metal release rate. The concentration of SlyD was then adjusted for the amount bound to HypB, assuming 1:1 binding, to yield the free SlyD concentration. These data were then fit to a saturation equation, $k_{\text{off}} = k_{\text{off}}^{\text{max}}([\text{SlyD}])/(K_d + [\text{SlyD}])$.

Molecular Chaperone Assays—The ability to suppress aggregation of citrate synthase (CS) was used as an assay of general molecular chaperone activity (40). Chemically denatured, reduced CS was prepared by desalting an amion sulfate preparation (Sigma) that was first diluted 4-fold with 50 mM Tris, pH 8.0, by using a PD-10 column pre-equilibrated with 50 mM Tris, pH 8.0. CS-containing fractions were pooled, concentrated, and adjusted to 40 μM based on the monomeric mass of 48,969 Da and the published extinction coefficient at 280 nm of 1.78 absorbance units for a 1 mg/ml solution (40). The CS stock was chemically denatured and reduced by diluting the protein 4-fold in 6 mM guanidinium hydrochloride and 25 mM dithiothreitol and incubating at room temperature for 2 h. For each aggregation assay 5 μl of CS were diluted with 500 μl of buffer A (to a final concentration of 0.1 μM) in the presence or absence of SlyD or a SlyD variant at a 20:1 ratio (2 μM). Aggregation was monitored on a fluorimeter (Jolin Yvon) with $\lambda_{\text{ex}}$ and $\lambda_{\text{em}}$ both set at 500 nm and slit widths set at 2 nm.

To assay for CS reactivation, 2 μl of CS were diluted in 100 μl of buffer A (to a final concentration of 0.2 μM) in the presence or absence of SlyD or a SlyD variant at a 20:1 ratio (4 μM). After 2 h, a 2-μl aliquot was removed and assayed for CS activity by diluting into 98 μl of reaction mixture (93 μl of 50 mM Tris, 2 mM EDTA, pH 8.0, 3 μl of 5 mM acetyl-CoA, 1 μl of 10 mM oxaloacetic acid, and 1 μl of 10 mM DTNB). The reaction was monitored at 412 nm because of the reaction of CoASH with DTNB, and the initial rate was compared with that of a control reaction performed with an equivalent amount of untreated CS, defined as 100% activity.

Chemical Cross-linking—Purified HypB was incubated with nickel followed by gel filtration, and the extinction coefficients at 320 nm were within 5% of the listed values.

Cell Strains—The ΔslyD mutation in a DY330 background (41) was described previously (19). The ΔslyD cells in the MC4100 background (42), used for the experiments shown in Fig. 4C, were prepared in the same manner. The ΔhypB strain (DHP-B) in the MC4100 background was generously provided by Prof. A. Böck (University of Munich, Germany (43)). Protein expression from the pBAD plasmids was induced with 1 and 100 μM arabinose in the MC4100 and DY330 strains, respectively, unless otherwise noted.

Growth Condition and Preparation of Crude Cell Extracts—Cells were grown aerobically in LB media overnight prior to anaerobic growth in TGYEP (44) supplemented with 1 mM sodium selenite, 0.8% glycerol, and 15 mM sodium fumarate as well as arabinose and NiSO$_4$ at the indicated concentrations. Cell extracts were prepared as described previously (19) and tested for hydrogenase activity under a H$_2$(g)-containing atmosphere by using benzyl viologen as an electron acceptor according to the method of Ballantine and Boxer (45).

Western Blotting—Crude cell extracts were separated by SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and incubated with either a 1:1,000 dilution of anti-HypB (from Prof. A. Böck) or anti-SlyD polyclonal antibodies (prepared by immunization of rabbits with purified SlyD, Division of Comparative Medicine, University of Toronto), followed by incubation with a goat anti-rabbit-horseradish peroxidase secondary antibody (Bio-Rad). Enhanced chemiluminescence (Pierce) was used for detection.

RESULTS

SlyD, but Not SlyDΔflap, Suppresses the Aggregation of Citrate Synthase (CS)—Many PPIases exhibit molecular chaperone activity that is distinct from the PPLase activity, but some are chaperones of only a limited set of client proteins (reviewed in Ref. 23). To determine whether SlyD acts as a general molecular chaperone, experiments were performed to examine if full-length SlyD is capable of preventing the aggregation of chemically denatured, reduced CS. As shown in Fig. 2A, SlyD almost completely suppresses aggregation at a ratio of 20:1. The same in vitro assay was recently used to demonstrate that a truncated SlyD lacking the C-terminal tail, SlyD(1–165), acts as a molecular chaperone (26). The SlyD variant lacking the complete C-terminal tail, SlyD(1–146), has a similar activity as that reported for SlyD(1–165) (26) and is partially impaired in comparison with full-length SlyD (Fig. 2B). This result indicates that the tail does play a limited role in the molecular chaperone activity.

SlyD, like some of the other FKBP's, possesses an additional IF domain that may bind to substrates with an extended conformation (27, 28). To examine whether the IF domain has a role in the ability of SlyD to act as a molecular chaperone, we engineered a variant lacking residues 107–111 (referred to as SlyDΔflap). This deletion, based on sequence similarity with
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To confirm the chaperone capabilities of SlyD, experiments were performed to determine whether the protein would influence productive folding of CS. The refolding yield of CS is significantly enhanced in the presence of a 20-fold excess of SlyD, increasing to 68% reactivation from the 32% spontaneous refolding (Fig. 2C), a level of enhancement equivalent to that observed in the presence of a 1:1 ratio of GroEL/GroES + ATP (40). In correspondence with the aggregation suppression experiments, SlyD-(1–146) promotes refolding of CS to a lesser degree than full-length SlyD, and SlyD(Δflap) has an effect that is only slightly above basal levels of spontaneous reactivation.

SlyD(Δflap) Does Not Cross-link with HypB in Vitro or Activate Hydrogenase in Vivo—As the IF domain is a potential mediator of protein-protein interactions, mutations in this domain that result in compromised molecular chaperone activity might also affect binding to specific partner proteins. The fact that SlyD(Δflap) failed to suppress aggregation or reactivate CS suggested that this variant did not interact with the folding intermediates. To test if this deletion in SlyD also disrupts the complex with the hydrogenase accessory protein HypB, a chemical cross-linking assay was used. As shown previously, the addition of EDC, a water soluble, zero-length cross-linker, to preincubated HypB and SlyD results in a robust cross-link that migrates at the molecular weight of the heterodimer on a denaturing polyacrylamide gel (Fig. 3A) (19). A cross-linked dimer is, however, not observed between HypB and SlyD(Δflap), indicating that this loop from the SlyD IF domain is required for complex formation with HypB. In agreement with this conclusion, removal of the C-terminal tail of SlyD (SlyD-(1–146)) did not prevent the interaction with HypB (Fig. 3B).

To determine whether one or both of the properties of SlyD disrupted in the SlyD(Δflap) variant, folding chaperone activity or HypB binding, are required for its function in hydrogenase biosynthesis, ΔslyD cells were transformed with pBAD24, pBAD-SlyD, or pBAD-SlyD(Δflap). The hydrogenase activity in the cell lysates of the transformed cells was measured by using benzyl viologen as a chromophoric electron acceptor and com-

the structurally characterized MtFKBP17 (27), is predicted to remove a short loop near the putative polypeptide-binding site in the IF domain. SlyD(Δflap) retains 50% of wild-type PPIase activity and has a CD spectrum similar to that of wild-type SlyD (data not shown), ruling out the possibility that this deletion results in loss of structure. However, a test of SlyD(Δflap) in the aggregation assay with chemically denatured CS revealed that this mutant is severely impaired in its ability to prevent aggregation compared with wild-type SlyD (Fig. 2B).

SlyD, but Not SlyD(Δflap), Can Promote CS Reactivation—Upon dilution from denaturant, CS can refold to form active enzyme, but the competing aggregation pathway limits the yield of active CS. In the absence of added chaperone, unfolded CS undergoes a time-dependent refolding to a maximum of "~30% initial activity (40). To confirm the chaperone capabilities of SlyD, experiments were performed to determine whether the protein would influence productive folding of CS. The refolding yield of CS is significantly enhanced in the presence of a 20-fold excess of SlyD, increasing to 68% reactivation from the 32% spontaneous refolding (Fig. 2C), a level of enhancement equivalent to that observed in the presence of a 1:1 ratio of GroEL/GroES + ATP (40). In correspondence with the aggregation suppression experiments, SlyD-(1–146) promotes refolding of CS to a lesser degree than full-length SlyD, and SlyD(Δflap) has an effect that is only slightly above basal levels of spontaneous reactivation.

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FIGURE 4. Hydrogenase activity of E. coli expressing HypB and SlyD variants. A, wild-type (DY330), ΔslyD (DY330), and ΔslyD cells transformed with pBAD, pBAD-SlyD, pBAD-SlyD(1–146), or pBAD-SlyD(DΔflap) were grown anaerobically in TGYEP with 100 μM arabinose for 6 h. Cell extracts were prepared and tested for hydrogenase activity by using benzyl viologen reduction. The rates of benzyl viologen reduction were adjusted for total protein concentration, and the data were averaged and then normalized to the value for wild-type (WT) cells. Asterisks indicate data that are different at the 99% confidence level from those generated with pBAD-SlyD as determined by using a Student’s t test. B, wild-type (MC4100), ΔhypB(MC4100), and ΔhypB cells transformed with pBAD, pBAD-HypB, pBAD-HypB(295,P325), pBAD-HypB(26d36), or pBAD-HypB(11d76) were grown anaerobically in TGYEP, 5–10 μM Ni(II), and 1 or 10 μM (pBAD-HypB(11d76) arabinose, and tested for hydrogenase activity as above. Asterisks indicate data that are different at the 99% confidence level from those generated with pBAD-HypB as determined by using a Student’s t test. C, wild-type (MC4100), ΔslyD, and ΔhypB prepared from the MC4100 strain, and ΔhypB cells transformed with pBAD-HypB or pBAD-HypB(11d76) were grown as above in the absence (gray bars) or presence (black bars) of extra 300 μM Ni(II) in the growth media, and cell extracts were tested for hydrogenase activity. Asterisks indicate data from cells grown in extra nickel that are different at the 95% confidence level from those generated with the same cells grown in the absence of nickel as determined by using a Student’s t test. Error bars indicate ± 1 S.D.

pared with the activity in extracts from wild-type cells. The ΔslyD cell extracts had an activity that was only about 25% of the parent cells as reported previously (19), demonstrating that SlyD plays a role in the activation of hydrogenase but is not absolutely essential (Fig. 4A). Wild-type levels of activity can be restored by the transformation of ΔslyD cells with pBAD-SlyD, but no restoration is achieved by transformation with pBAD-SlyD(DΔflap), indicating that either the molecular chaperone activity of SlyD or its ability to bind HypB is required for the hydrogenase activation pathway. Western analysis of the cell extracts probed with an anti-SlyD polyclonal antibody confirmed that the expression of both SlyD and SlyD(DΔflap) from the pBAD vector was at levels comparable with SlyD expression in DY330 control cells (data not shown). The results for SlyD-(1–146) in the hydrogenase assay will be discussed below.

HypB Linker Region Participates in Complex Formation with SlyD—To localize the site of interaction with SlyD on HypB, a chemical cross-linking experiment was first performed with SlyD and the isolated GTPase domain of HypB (residues 77–290). A cross-link between the HypB GTPase domain and SlyD was not observed, and the addition of an excess of the GTPase domain did not result in a reduction in the amount of cross-link between full-length HypB and SlyD (data not shown). This result suggested that SlyD binds to the N-terminal region of HypB.

As a PPlase, SlyD catalyzes isomerization in vitro on model substrates with a hydrophobic residue preceding the target proline (29) and in the sequence linking the N-terminal CXXCGC motif and the GTPase domain there is a proline-containing sequence, 28APFAPAARP36 (see Fig. 1). To assess whether this sequence is involved in the SlyD-HypB interaction, serine was substituted for the first two (underlined) of the three prolines to yield HypB(P29S,P32S). This mutation resulted in a reduced amount of cross-linked complex with SlyD (Fig. 3C) supporting the hypothesis that SlyD interacts with HypB at least partially via this proline-containing motif. A similar result was observed for a HypB(28d36) mutant, which has the sequence between residues 28 and 36 deleted. Furthermore, proteins prepared with more substantial linker deletions, HypB(19d76) and HypB(11d76), do not produce any cross-link with SlyD under our standard conditions, indicating that there is an additional site of interaction beyond the 28APFAPAARP36 sequence within this linker region (Fig. 3C and data not shown). All of the HypB variants bound stoichiometric nickel with high affinity (Table 2), were as active as wild-type HypB in the GTPase assay within experimental error, and exhibited secondary structure content similar to HypB (HypB(P29S,P32S) and HypB(28d36)) or the HypB GTPase domain (HypB(19d76) and HypB(11d76)) as assessed by CD spectroscopy (data not shown).

Impaired SlyD-HypB Complex Formation In Vitro Correlates with Decreased Hydrogenase Activation In Vivo—To assess the importance of the HypB linker region in the hydrogenase metallocenter assembly pathway, arabinose-inducible plasmids expressing HypB(P29S,P32S), HypB(28d36), or HypB(11d76) were transformed into ΔhypB cells. Extracts from ΔhypB cells transformed with pBAD-HypB and grown under the same conditions had a relative hydrogenase activity of ~80% compared with the control cells, whereas ΔhypB cells had no hydrogenase activity as expected (Fig. 4B) (43). Hydrogenase activities measured in extracts from cells expressing HypB with the Pro-Ser mutations (pBAD-HypB(P29S,P32S)) or the 28APFAPAARP36 sequence removed (pBAD-HypB(28d36)) were lower than for pBAD-HypB-transformed cell extracts, suggesting that hydrogenase activation is compromised by disrupting the binding of SlyD to HypB. Under the standard conditions of induction with 1 μM arabinose, cell extracts from pBAD-HypB(11d76) cells had negligible hydrogenase activity, but unlike the other HypB variants, Western blotting indicated that the HypB(11d76) was poorly expressed (data not shown). Increasing the arabinose concentration from 1 to 10 μM resulted in protein expression at
A level similar to that of wild-type HypB in the MC4100 cells. These HypB(11d76) cell extracts exhibited limited activity (Fig. 4B), suggesting that the interaction of SlyD with the linker region of HypB plays a role in full hydrogenase activation.

Nickel Complements HypB Mutant—The weak hydrogenase activation of HypB(11d76) could result from this HypB variant blocking Ni(II) insertion or failing to release its bound Ni(II). As has been reported previously, the addition of excess nickel to the growth media can complement to a small degree the hydrogenase-deficient phenotype of the ΔhypB cells (Fig. 4C) (10) and, to a greater degree, the ΔslyD cells (Fig. 4C) (19). Although the hydrogenase activities of wild-type and pBAD-HypB cells are not affected by the addition of nickel, the activity of pBAD-HypB(11d76) cells increases significantly (Fig. 4C), suggesting that the loss of interaction between HypB and SlyD can be compensated partially by the addition of nickel to the growth medium.

SlyD Stimulates Metal Release from HypB—To investigate the consequences of the interaction between SlyD and HypB, a kinetic metal release assay was employed. In this experiment released metal is captured by 4- (2-pyridyldiazol)resorcinol (PAR), a chromophoric metal chelator that is used to monitor metal release from proteins (39). We have previously used this assay to show that nickel is slowly released from the high affinity site of HypB ($t_{1/2} \approx 22$ h; Fig. 5A) (14). In this study, the PAR assay was used to evaluate the effect of SlyD on nickel binding to HypB. The addition of increasing amounts of SlyD results in a dramatic increase in the rate of metal release from HypB, with the effect saturating at a SlyD:HypB ratio of about 10:1 and a $t_{1/2} \approx 20$ min (Fig. 5A). A plot of the observed rates of metal release versus SlyD concentration yields a saturation curve with a $K_d$ for the HypB-SlyD interaction of $9\, \mu M$, assuming a 1:1 binding stoichiometry (Fig. 5B). These results reveal that SlyD is catalyzing metal release from HypB. To rule out the possibility of protein degradation during the assay, a sample of HypB was incubated with SlyD for 2 h at room temperature and subjected to SDS-PAGE (data not shown). To test the possibility that SlyD was causing metal release by using a redox mechanism, a reaction was setup in an anaerobic glove box (96% nitrogen, 4% hydrogen), and SlyD-stimulated metal release from HypB was still observed. Metal release under the standard aerobic conditions was also unaffected by the addition of a reducing agent (1 mM TCEP) to the reaction buffer. Preincubation of HypB and SlyD for 2 h before the addition of PAR did not result in an immediate significant signal at 500 nm, indicating that PAR acts to sequester the metal from the proteins once it is released from the high affinity site of HypB.

Metal Release Depends on HypB-SlyD Complex Formation—To determine whether the physical interaction between HypB and SlyD is required for faster metal release from HypB, the HypB and SlyD variants described above with defective complex formation in the chemical cross-linking assay were examined. The addition of SlyD(Δflap) results in slower metal release from HypB compared with the reaction with wild-type SlyD at the same SlyD:HypB ratio of 10:1 ($t_{1/2} > 5$ h compared with $t_{1/2} \approx 20$ min; see Fig. 6A). Similarly, an examination of the HypB linker region variants revealed a correlation between complex formation with SlyD and SlyD-induced metal release (Fig. 6B). The addition of SlyD to HypB(P29S,P32S) stimulated metal release but not as dramatically as from wild-type HypB ($t_{1/2} \approx 1$ h). Metal release was even slower from HypB(28d36) ($t_{1/2} \approx 2$ h) and very weak from HypB(19d76) and HypB(11d76) ($t_{1/2} \approx 5$ and 6 h, respectively). The metal

![FIGURE 5. SlyD stimulates metal release from HypB to PAR.](image)

A. HypB (5 μM) containing close to stoichiometric amounts of nickel was incubated with 100 μM PAR (closed circles) and the indicated amounts of SlyD. Metal release was monitored by measuring the absorbance at 500 nm of the metal-PAR complex. The data were converted to percent metal bound by determining total metal content following treatment of a separate HypB sample with 100 μM parahydroxymercuribenzoic acid, and then fit to an exponential decay equation. B. first-order rate constants of metal release from HypB in the presence of increasing concentrations of SlyD were fit to a saturation equation with a $K_d$ value of 9 μM.

![FIGURE 6. Variants deficient in SlyD-dependent metal release from HypB to PAR.](image)

A. HypB (5 μM) was incubated with 100 μM PAR in the presence of 1:1 SlyD, SlyD(Δflap), MBP-SlyD-(140–196), or SlyD-(1–146). B. HypB, HypB(P29S,P32S), HypB(19d76), or HypB(11d76) (5 μM) was incubated with 100 μM PAR in the presence or absence of 10:1 SlyD. For both plots metal release was monitored by measuring the absorbance at 500 nm because of the metal-PAR complex, and the data were treated as described for Fig. 5A.
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release reactions were repeated at a lower SlyD to HypB variant ratio (4:1 instead of 10:1), and SlyD barely stimulated metal release from HypB(19d76) and HypB(11d76) (∼10% released after 2 h compared with the 90% release observed for the same ratio of SlyD with wild-type HypB), confirming that SlyD-stimulated release from these variants is greatly impaired (Fig. 6A and data not shown). An assessment of the nickel-binding activities of the four HypB variants demonstrated that HypB(19d76) and HypB(11d76) exhibit an increase in their ε₃₅₀ values (Table 2), although the profile of the entire spectra remains the same (data not shown), possibly indicative of a change in the ligand environment. Clearly, however, all four variants retain high affinity Ni(II) binding activity (Table 2) and exhibit slow metal release kinetics in the absence of SlyD (data not shown).

The C-terminal Tail of SlyD Is Required in Vivo for Hydrogenase Activation—Given that the C-terminal tail of SlyD is rich in metal-binding residues and that the protein can bind nickel ions (29), it is reasonable to suggest that this region of the protein is an important component of SlyD function in hydrogenase production, with a possible role in nickel storage. In support of this hypothesis, the hydrogenase activity of extracts from ΔslyD cells expressing SlyD-(1–146) was the same as the activity in the ΔslyD cells, indicating that the C-terminal tail is required for SlyD to function in the hydrogenase activation pathway (Fig. 4A).

The C-terminal Tail of SlyD Is Necessary but Not Sufficient to Stimulate Metal Release from HypB—The C-terminal tail of SlyD is involved with metal-dependent inhibition of PPase activity (29), and it influences the molecular chaperone activity of SlyD (Fig. 2), so even though this domain of SlyD is not required for complex formation with HypB (Fig. 3B), it remained possible that it could also modulate metal release from HypB. For this reason, SlyD-(1–146) was also tested in the metal release assay, and only a very slow release of metal from HypB was observed (t½ > 4 h; Fig. 6A). SlyD-(1–146) retains 60% of wild-type SlyD PPase activity (see Ref. 29 and data not shown) but has a significantly altered CD spectrum compared with SlyD. This suggests that the C-terminal tail may interact and stabilize the PPase domain to some degree. To investigate whether the tail on its own was sufficient to activate metal release, we constructed MBP-SlyD-(140–196), and this fusion protein had no effect on metal release from HypB (t½ = 24 h; Fig. 6A). To assess whether the cysteine residues of the C-terminal tail were required to stimulate metal release, SlyD was treated with an excess of iodoacetamide followed by dialysis. This treatment fully modified the six cysteine residues of the C-terminal tail as monitored by mass spectrometry, but the protein was still capable of stimulating metal release from HypB (data not shown).

DISCUSSION

SlyD derives its name from the observation that it is required for E. coli sensitivity to lysis mediated by the phage dX174 protein E (21), possibly by stabilizing the E protein through direct interactions (46, 47). However, in consideration of its physiological function, the C-terminal metal-binding domain and its ability to bind metal ions led to the hypothesis that SlyD was involved in E. coli metal homeostasis (21, 29). Subsequent studies revealed that SlyD does play such a role by contributing to nickel accumulation and nickel delivery to the apohydrogenases (19). These experiments suggested that SlyD directs nickel to the hydrogenase biosynthetic pathway as a result of its interaction with HypB. In vitro experiments were also used to demonstrate that SlyD is a PPase (29) and a molecular chaperone (26), but the roles of these activities in the biosynthesis of hydrogenase were not assessed.

The SlyD variant SlyD(Δflap), which lacks a five-residue loop embedded in a putative molecular chaperone domain, is impaired in its ability to suppress the aggregation and promote the reactivation of CS, a model protein folding substrate. Furthermore, in a screen of spontaneous mutations the same deletion in SlyD produced resistance to the phage dX174 lysis (48). In the homologous MtFKBP17, this loop is part of the IF domain that is required for the chaperone activity but not the PPase activity of the protein (28). The structure of MtFKBP17 revealed a hydrophobic surface on the IF domain (27), proposed to interact with substrates and prevent aggregation. This function of the IF domain is supported by the behavior of SlyD(Δflap) in the CS assays as well as by the fact that this variant fails to interact with HypB in vitro and is unable to support the activation of hydrogenase in vivo.

On the HypB side of the SlyD-HypB complex the protein-protein contacts lie in the linker region between the NH₂-CXX-CGC motif and the GTPase domain. The decrease in the interaction observed between SlyD and HypB(P29S,P32S) and the lack of a detectable interaction with HypB(11d76) suggest a proline-directed interaction with additional binding determinants in the linker region. The deficiency in complex formation in vitro correlates with reduced hydrogenase biosynthesis in vivo, which can be counteracted by excess nickel in the growth medium. These results support the hypothesis that a physical interaction between SlyD and HypB is required for the role of SlyD in Ni(II) insertion into apohydrogenase.

HypB has two Ni(II)-binding sites of vastly different affinities, one in the GTPase domain that has a micromolar Kd and one involving the N-terminal CXXCGC motif and the GTPase domain. The decrease in the interaction observed between SlyD and HypB(P29S,P32S) and the lack of a detectable interaction with HypB(11d76) suggest a proline-directed interaction with additional binding determinants in the linker region. The deficiency in complex formation in vitro correlates with reduced hydrogenase biosynthesis in vivo, which can be counteracted by excess nickel in the growth medium. These results support the hypothesis that a physical interaction between SlyD and HypB is required for the role of SlyD in Ni(II) insertion into apohydrogenase.

The C-terminal tail of SlyD is not absolutely required for the molecular chaperone activity of SlyD, but it does enhance it, perhaps by acting as a hydrophilic lid to the polypeptide-binding site or merely increasing the solubility of complexes with denatured substrates. This domain is also not essential for the interaction between SlyD and HypB in vitro, but it is critical for the role of SlyD in hydrogenase biosynthesis. At first glance, this...
result implies that the putative metal storage capacity of SlyD, localized to the tail with its numerous histidine, cysteine, and carboxylate residues, is just as critical for hydrogenase activation as the ability to bind to HypB. However, the SlyD variant lacking the C-terminal tail is also deficient in the in vitro simulation of metal release from HypB. Thus it is likely that the in vivo role of the SlyD tail is more than just metal storage, and one possible explanation is that it mediates a more accessible conformation of the Ni(II) high affinity site of HypB. In analogy, just as SlyD catalyzes in vitro transfer to the indicator PAR, this SlyD-stabilized open conformation of HypB could also allow nickel collection by the coordinating cysteines of the hydrogenase enzymes in vivo, thus facilitating nickel delivery. Several questions that remain to be resolved include whether a metal-binding site on SlyD itself or its PPIase activity are involved in the metal transfer from HypB.

Although none of the other members of the PPlase superfamily have a metal-binding domain like SlyD, some have been implicated in metal homeostasis. For example, FKBP52 binds to Atox1, a metallochaperone that plays a role in delivering copper for export by the Wilson and Menkes proteins, and overexpression of FKBP52 enhances copper release in a cell culture model system (49). There are other proteins that are involved in metal homeostasis and that have polyhistidine regions. In E. coli, RcnA (formerly YohM) functions as a nickel/cobalt efflux transporter and contains a polyhistidine stretch as part of a predicted cytoplasmic loop (50). Some homologs of the accessory protein UreE, required for the metallocenter assembly of nickel-containing urease, also possess a polyhistidine region, although this region is not essential for enzyme production (3). Unlike the C-terminal tail of SlyD, however, these regions in RcnA and UreE are not rich in acidic residues or cysteine. In contrast, the two Helicobacter pylori proteins HspA and Hpn have sequences rich in a mix of metal-binding residues that can bind multiple nickel ions (51–53). It is interesting to note that in this organism, which depends on both hydrogenase and urease for efficient colonization (54), the C terminus of the SlyD homolog has 3-fold fewer histidine residues than the E. coli protein, but whether the other nickel-binding proteins can functionally replace SlyD is not known.

The hydrogenase activity observed for cells expressing pBAD-HypB(11d76) resembles that observed for the SlyD knock-out cells and is significantly above that observed for ΔhypB cell extracts. Given the disruption of the HypB-SlyD interaction, this phenotype suggests that SlyD only contributes to hydrogenase biosynthesis through its interaction with HypB. SlyD is still present in ΔhypB cells expressing HypB(11d76), so the diminished hydrogenase activity cannot be due to just a loss of the Ni(II) storage function of SlyD but rather a loss of nickel delivery and/or to a loss of SlyD-stimulated metal release from HypB. The fact that the lack of an interaction between HypB(11d76) and SlyD does not result in a complete inability to activate hydrogenase, in contrast to the ΔhypB cells, indicates that SlyD-stimulated metal release is not the only route of nickel addition through HypB, but it may be the privileged pathway under normal growth conditions. This model is supported by the observation that the hydrogenase activity in the cells expressing HypB(11d76) can be significantly restored by excess nickel in the growth media. Because HypB(11d76) can still bind Ni(II) with high affinity, this result suggests that the addition of Ni(II) to the media bypasses the need for SlyD-stimulated Ni(II) release. This extra nickel may be able to reach the HypB mutant from a pool of nickel separate from the hydrogenase-directed supply of nickel employed in the absence of excess metal (55). Another possibility is that in addition to compromising the interaction with SlyD, HypB(11d76) fails to interact properly with the hydrogenase large subunit, resulting in impaired nickel insertion. This issue will have to be resolved in future studies.

In contrast, the minimal amount of activity restored in the ΔhypB cells grown in excess nickel, unlike the linker deletion mutants, suggests that HypB has a critical role for nickel insertion that is independent of SlyD. This function may be related to the essential GTPase activity of HypB (10), which is not disturbed in the variants analyzed. One could propose a model in which GTP hydrolysis, rather than playing a role in Ni(II) insertion per se, is linked to a subsequent quality control step that drives a conformational change releasing the bound accessory proteins HypA, HypB, and HypC from the large subunit of hydrogenase. Such a role would not require a tight or specific metal-binding site in the GTPase domain, because protein–protein interactions could maintain HypB in proximity with the metallocenter of hydrogenase. Indeed, the metal–binding site in the GTPase domain of HypB has a Kd value only in the micromolar range and is not selective for Ni(II) over Zn(II) (14). A recent x-ray crystal structure of HypB from Methanocaldococcus jannaschii shows that the metal-binding residues are intimately linked to the GTPase active site, suggesting a possible triggering mechanism whereby metal binding could stimulate GTP hydrolysis (18). Supporting this hypothesis is the fact that the low affinity metal-binding site of HypB is required for hydrogenase activation. HypB also dimerizes in solution (this work and see Refs. 19 and 35), but the role of HypB dimerization in hydrogenase maturation, as opposed to complex formation with SlyD, has not been assessed. Now that the players involved have been identified and characterized, the intriguing molecular details of the metal transfer pathway for nickel addition to hydrogenase can be examined.

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