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[NiFe]-Hydrogenase Maturation

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Abstract

[NiFe]-hydrogenases catalyze the reversible conversion of hydrogen gas into protons and electrons and are vital metabolic components of many species of bacteria and archaea. At the core of this enzyme is a sophisticated catalytic center comprising of nickel and iron, as well as cyanide and carbon monoxide ligands, which is anchored to the large hydrogenase subunit through cysteine residues. The production of this multi-component active site is accomplished by a collection of accessory proteins and can be divided into discrete stages. The iron component is fashioned by the proteins HypC, HypD, HypE, and HypF, which functionalize iron with cyanide and carbon monoxide. Insertion of the iron center signals to the metallochaperones HypA, HypB, and SlyD to selectively deliver the nickel to the active site. A specific protease recognizes the completed metal cluster and then cleaves the C-terminus of the large subunit, resulting in a conformational change that locks the active site in place. Finally, the large subunit associates with the small subunit and the complete holo-enzyme translocates to its final cellular position. Beyond this broad overview of the [NiFe]-hydrogenase maturation process, biochemical and structural studies are revealing the fundamental underlying molecular mechanisms. Here, we review recent work illuminating how the accessory proteins contribute to the maturation of [NiFe]-hydrogenase and discuss some of the outstanding questions that remain to be resolved.

Introduction Hydrogenases are enzymes that use intricate metallic active sites to catalyze the interconversion between hydrogen gas and protons and electrons.[1-3] These enzymes are found in organisms from all three domains of life that inhabit diverse environmental niches around this planet. The production and consumption of H_2 gas by hydrogenases have critical roles in the global H_2 cycle and are intimately connected to the nitrogen and carbon cycles.[3-5] There are three phylogenetically distinct classes of hydrogenases, each with a different type of metallocenter at the active site, called [NiFe]- , [FeFe]-, and [Fe]-hydrogenases.[2, 6] The [NiFe]-hydrogenase class has a bimetallic catalytic core that is buried deep in the larger subunit of a heterodimeric protein, and is composed of an iron bound to two cyanide ligands, one carbon monoxide ligand, and two cysteine residues that bridge to a nickel ion, which is further coordinated to two additional cysteine residues (Figure 1). The small subunit contains iron-sulfur clusters that are used to transport electrons towards or away from the active site.

[NiFe]-hydrogenases are found in many taxa of bacteria and archaea where, depending on the cellular context, they can oxidize H_2 as a source of energy for the organism or produce the gas as a means of disposing of excess reducing equivalents and of conserving energy.[2, 3, 7] There are a variety of ways for these enzymes to contribute to cell metabolism, and they are typically located at the cytoplasmic membrane in association with multi-protein membrane complexes.[2, 8-11] In addition to the fundamental role that these enzymes play in biology, [NiFe]-hydrogenases are of interest because the key positions that they hold in several human pathogens make them potential drug targets,[12-15], as well as the promising biotechnology applications in the production of renewable energy and in bioremediation.[1, 2]

[NiFe]-hydrogenases can be divided into multiple subgroups based on sequence and function, as well as on special properties such as the degree of oxygen tolerance for catalytic

activity.[1, 2, 7] Despite these distinctions, it appears that the core metallocenter is preserved between subgroups. For example, protection from oxygen is provided by strategies independent of the active site, such as modifications to the iron-sulfur clusters in the small subunit or reduced gas accessibility to the catalytic center through constricted gas channels.[16-18] In one notable exception, several subgroups of the [NiFe]-hydrogenases are produced with one of the terminal nickel-coordinating cysteines replaced with selenocysteine.[19] Although [NiFeSe]-hydrogenases can lose activity upon exposure to oxygen, they are quickly reactivated, resulting in a tolerance that makes them particularly attractive for biotechnological applications.[1, 16, 19]

Unsurprisingly, the production of [NiFe]-hydrogenases is a complex process, involving a suite of maturation proteins that perform a variety of tasks that include the generation of the small molecule iron ligands and the insertion of nickel into the active site.[20-22] The same maturation proteins are typically found in all of the organisms that produce [NiFe]-hydrogenases, but there are variations in the details of how this process is accomplished. To elucidate the biosynthetic pathway of the [NiFe]-hydrogenase enzymes, a battery of methods drawn from biochemistry, spectroscopy, structural biology, and microbiology have been applied. Much of the current information on [NiFe]-hydrogenase maturation is from studies of *Escherichia coli*, so this organism will provide the basis for this review, supplemented by information from other organisms. *E. coli* produces at least three [NiFe]-hydrogenase enzymes, referred to as hydrogenase 1, 2 and 3. Most of the [NiFe]-hydrogenase maturation genes are named *hyp*, which stands for "genes affecting hydrogenases pleiotropically".[3, 9] In brief, HypC, HypD, HypE and HypF are responsible for the production and delivery of the iron center along with its diatomic ligands, whereas HypA and HypB are required for nickel insertion.[20-22] Although many components of the maturation machinery are involved in the production of all of the *E. coli*

hydrogenases, HypA and HypC are required for the production of hydrogenase 3 and are functionally replaced by HybF and HybG, respectively, during biosynthesis of the other hydrogenases.[23, 24] An enzyme-specific protease finishes the maturation process by removing a peptide from the end of the C-terminus. Production of the large subunit is paralleled by concurrent maturation of the small subunit, and once matured these two subunits combine and are transported to their final destination, where they associate with other proteins to complete the functional systems.[3, 22] The topic of this review is the extraordinary manner in which [NiFe]-hydrogenases are produced with particular focus on nickel insertion into the bimetallic active site.[20-22]

Iron Insertion

Staged Process. The sequence of events that occur to produce [NiFe]-hydrogenases can be divided into several discrete stages, including sequential delivery of iron followed by nickel. The model that iron is delivered first was suggested by the observation that nickel is not inserted if the iron accessory proteins are deficient,[25] and supported by isolation of a partially-completed hydrogenase large subunit from *Ralstonia eutropha* that was fully loaded with iron but not nickel.[26, 27] This stepwise progression suggests that there are checkpoints along the way to certify that each stage is completed before the next one begins. Furthermore, stepwise construction of the metal center implies that the active site is assembled directly on the enzyme precursor protein, in contrast to the maturation of many other metalloenzyme clusters, which are often constructed on a scaffold protein before delivery.[28-30] Interestingly, some organisms possess HupK, a protein similar to the [NiFe]-hydrogenase large subunit that appears to serve as a platform for metallocenter assembly in a manner that protects the cofactor from oxygen.[22]

Biosynthesis of the Diatomic Ligands. In addition to the two metal ions, the active site contains both carbon monoxide and cyanide coordinated to the iron.[1, 6] Although the other classes of hydrogenase enzymes have different phylogenetic lineages, the presence of the diatomic ligands is conserved across all known types in what is considered to be convergent evolution,[1, 2, 6] underscoring the key role that these ligands play in the reaction catalyzed by hydrogenases. However, the biosynthesis of these toxic molecules is accomplished by different strategies for each of the hydrogenase classes. For the production of the [NiFe]-hydrogenases, the Hyp accessory proteins transform carbamoyl phosphate into cyanide.[31, 32] In contrast, isotopic labeling studies have revealed that the carbon monoxide is generated for the enzyme from an intracellular precursor that may stem from glycerol, but currently the process remains undefined.[31-34] HypC and HypG isolated from *E. coli* contain sub-stoichiometric iron and exhibit an infrared spectroscopic signal for CO_2,[35, 36] hinting at a possible precursor that could be reduced to form the carbon monoxide complex. However, this intermediate must be metabolically generated, as exogenously added bicarbonate was not a source of carbon monoxide for the [NiFe]-hydrogenase in *R. eutropha*.[32] Besides the outstanding question of how the CO is made, it is not clear how the precise stoichiometry of one carbon monoxide and two cyanide ligands is consistently maintained.

The activation of carbamoyl phosphate to produce cyanide is thought to occur via a transformation into an AMP adduct by HypF (Figure 2).[37] HypF then transfers the carboamide moiety onto the conserved C-terminal cysteine of HypE.[38-40] Structural analyses of HypF suggests that the protein has three distinct sites to carry out carbamoyl phosphate hydrolysis, formation of the adenylate intermediate, and transfer of the carboamide group to HypE.[41, 42] Each

of the three sites on HypF are connected by protein channels, possibly to minimize the loss of intermediates to solution. Furthermore, the structure of the HypE-HypF complex suggests that the C-terminus of HypE has close access to the final site on HypF (Figure 3).[40, 41, 43] The C-terminus of HypE appears to be flexible with respect to the rest of the protein and could alternate between being buried and being solvent exposed, such that it would be available to interact with other partner proteins to accept or deliver a protein modification.

When the C-terminus of HypE is buried within a homodimer, an internal ATP-dependent dehydration reaction that converts the carbamoyl modification into a thiocyanate has been observed.[38-40, 43, 44] The proposed dehydration mechanism proceeds through a phosphorylated intermediate flanked by two deprotonation steps.[39, 45] Recent spectroscopic analysis of HypE in a complex with HypF suggested the presence of an isothiocyanate adduct instead of thiocyanate, supporting a slightly different mechanism.[46] It is unclear whether this reaction has any biological relevance or how such an adduct would be employed *in vivo*.

Iron Center Assembly. Cyanide and carbon monoxide are added to an iron ion prior to its delivery to the [NiFe]-hydrogenase large subunit, and the sequence of events is summarized in Figure 2. To transfer the cyanide moieties to the nascent active site, HypE interacts with a HypD-HypC complex.[25, 47, 48] Isolated HypD-HypC (or the equivalent HypD-HybG) from several organisms contain both CN and CO ligands, suggesting that this complex serves as a scaffold for the preparation of the complete iron center.[36, 49-51] Incomplete iron centers on HypD-HypC suggest that cyanide loading is a prerequisite for CO addition onto the iron,[49] but spectroscopic evidence for a small amounts of Fe(I)-CO suggest that the diatomic ligands are added in the opposite order,[52] so the sequence of events is not yet clear. The proposed

mechanism for cyanide transfer onto the iron center requires two electrons to load each diatomic ligand onto the metal ion.[39] Analysis of the *Thermococcus kodakaraensis* proteins led to the suggestion that the electrons come from a redox cascade in HypD linking several disulfide bonds to the Fe_4S_4 cluster found in this accessory protein.[44, 47] This model is consistent with the observation that chemical modification of free thiols blocks CN transfer,[53] but does not account for the observation that one set of these cysteines is not conserved and is dispensable for HypD function.[49]

Another issue in this process is the origin of the iron ion for the [NiFe]-hydrogenase active site. It was initially suggested that the Fe_4S_4 cluster of HypD is the source of the hydrogenase iron,[47] but contemporary evidence suggests that the HypC-HypD complex contains an extra iron ion that is likely bound at the interface between the two proteins by several conserved cysteines, including the conserved N-terminal Cys2 of HypC (Figure 3).[36, 48] It is also possible that HypD alone may serve as a platform for the iron center prior to delivery to the hydrogenase enzyme, as it can be isolated bearing the spectroscopic signature of the complete iron center.[51]

Finally, the iron center must be delivered to the unprocessed hydrogenase large subunit (LS). HypC, or the functionally comparable HybG, form complexes with their respective LS,[23, 25, 54-56] so it is likely that they facilitate iron insertion. Analysis of HybG complexes revealed that it interacts with the hydrogenase LS along with HypD and HypE.[55] This observation raises the question of whether the iron center assembly occurs on accessory proteins docked at the destination site, although this quaternary complex was not observed in a homologous system.[56] The interaction of HypC/HybG with the LS depends on Cys2 of the putative chaperone,[23, 57] which also serves as an iron ligand within the HypC-HypD complex, suggesting that this residue

has a key role for iron center delivery. The complex between HypC and HycE (the large subunit of hydrogenase 3) also depends on Cys241 of HycE, which is a non-bridging nickel ligand in the final metallocenter.[57] In the completed active site, the four cysteines of the two conserved motifs, $C_{241}xxC_{244}$ and $C_{531}xxC_{534}$, coordinate the nickel with the second cysteine in each pair bridging to iron.[2] It is possible that HypC/HybG stabilizes the LS active site structure to accommodate insertion of both metal centers before releasing the LS and prompting a conformational change that releases the final cysteine ligand and buries the active site within the LS. Furthermore, there must be some change in the HypC-HycE complex that signals successful completion of the iron center, because the nickel accessory proteins only interact with the LS if the iron accessory proteins are functional.[58]

Nickel Delivery

Nickel insertion in the *E. coli* [NiFe]-hydrogenases is carried out by, at minimum, three metallochaperones: HypA, HypB and SlyD. All three accessory proteins were assigned to the nickel delivery stage of maturation because the hydrogenase deficiencies of knockout strains were complemented by supplementing the growth media with nickel.[59-66] The essential nature of the nickel metallochaperones underscores the competitive and limited nickel environment of a healthy cytoplasm. It is not known how much nickel is available in bacteria, but the concentrations of free nickel that activate the nickel metalloregulators that have been examined *in vitro* suggest that very little extra nickel is allowed to accumulate,[67, 68] which would minimize any repercussions of using this potentially toxic metal.[69] Thus, even though the nickel accessory proteins do not catalyze a chemical transformation during [NiFe]-hydrogenase production, they

are necessary because their environment obligates a dedicated nickel delivery system to ensure maturation of the metallocenter.

All three nickel metallochaperones, SlyD, HypA, and HypB were extracted along with the LS of hydrogenase 3 (HycE) from *E. coli* cell extracts by using pull downs,[58, 70] hinting that they function together in a cooperative complex to deliver nickel to the active site. The biochemical properties of HypA, HypB, and SlyD have been characterized individually,[67, 71] including the essential nickel-binding sites found on each protein. Furthermore, recent work on the complexes between the *E. coli* proteins suggests several possible pathways for selective nickel insertion into the hydrogenase enzyme precursor protein.

HypA. Escherichia coli produces both HypA and the homologous protein HybF, and these two forms of the cytosolic nickel metallochaperone are responsible for the maturation of different [NiFe]-hydrogenase enzymes produced by this organism.[72] One possible explanation for the presence of two versions of the same protein in *E. coli* is that HypA and HybF serve as adaptors that dock with their respective hydrogenase precursor proteins and act as scaffolds for the assembly of the other nickel accessory factors. In support of this model, *E. coli* HypA interacts directly with both HycE and HypB, and mediates the formation of a complex between the two other proteins.[58, 73]

Homologs of HypA from a variety of different organisms, including *E. coli, T. kodakaraensis,* and *Helicobacter pylori*, have been studied *in vitro* and the structures of the latter two were solved by X-ray crystallography and NMR (Figure 4), respectively.[74, 75] These data illuminated a protein comprised of two domains separated by a potentially flexible linker: a nickel-binding domain consisting of two alpha helices and three beta-strands, as well as a less-

ordered zinc-binding domain (Figure 4).[74, 75] HypA is found as both a monomer and a dimer, where the dimer form is mediated by strand swapping such that the ligands of each zinc site are supplied by both monomers.[73-76]

The zinc-binding domain of HypA contains a tetrathiolate zinc site composed of four cysteine residues from a conserved $CxxC(x)_nCPxC$ motif.[73, 75, 77] Zinc binds to this site with nanomolar affinity and as such, zinc is routinely pulled down with HypA during purification.[72, 73] This zinc motif, bound to multiple loops gathered together by three β-strands, is reminiscent of the structural zinc motifs found in proteins that mediate biomolecular interactions,[75, 78] and contains most of the residues that interface with HypB during HypA-HypB complex formation.[79] Furthermore, the variable sequence found in this region is consistent with the hypothesis that HypA and HybF interact selectively with their respective hydrogenase precursor proteins and facilitate the assembly of the other nickel accessory factors on the large subunits.

In addition to the structural role, it has been suggested that the zinc domain might contribute to the regulation of nickel use by *H. pylori* HypA (*Hp*HypA). In this organism, *Hp*HypA not only contributes to [NiFe]-hydrogenase maturation but also shuttles nickel to urease,[62, 80] another nickel-containing enzyme that is required for the survival of this pathogen in the acidic environment of the human stomach.[81, 82] Spectroscopic analysis of *Hp*HypA demonstrated that the zinc-binding site undergoes ligand substitution with histidine residues, depending on both pH and nickel loading, suggesting a regulatory role of the zinc site and communication between the two domains.[76, 83, 84] These changes could provide a signal that causes *Hp*HypA to divert nickel to urease during acid stress, an activity that may be a unique feature of the *H. pylori* homolog as the histidine residues proximal to the zinc site are not conserved across species. Recent *in vivo* characterization of *Hp*HypA mutants confirmed that the

cysteine residues are vital for urease maturation but demonstrated that the histidine residues are dispensable, so the role of histidine in this potential regulatory mechanism is not yet clear.[85]

The nickel-binding site of HypA has not been entirely defined, but a combination of mutagenesis and spectroscopic analysis revealed that it involves the side chains of two highly conserved residues, His2 and Glu3, as well as several additional N/O ligands including backbone nitrogens, to form an octahedral environment.[72, 74, 76, 80] Conversely, a recent crystal structure of *T. kodakaraensis* HypA (*Tk*HypA) revealed a square planar geometry containing a second histidine that is not conserved in the *E. coli* proteins.[79] The nickel-binding affinity of the *Tk*HypA homolog is dramatically increased only when the protein is in a complex with *Tk*HypB, a feature not yet observed in other HypA proteins,[79] so this protein system may provide a different mechanism of nickel delivery. Along the same lines, studies of various HypA homologs have reported a range of apparent nickel affinities that may point to discrepancies between methods or have roots in organism-specific nickel demands. For instance, *Ec*HypA has nanomolar affinity for nickel,[86] whereas *Hp*HypA cooperatively binds two nickel ions per dimer with micromolar affinity.[80] Nonetheless, it is clear that this site is critical for [NiFe]-hydrogenase maturation because disruption of HypA or HybF nickel binding via mutation of His2 causes a loss of hydrogenase activity and, in the case of *Hp*HypA, urease activity.[72, 80]

HypB. As with many metallocenter assembly pathways,[28, 29, 87] maturation of the [NiFe]-hydrogenase enzyme requires an NTPase, and this activity is the responsibility of the cytosolic metallochaperone HypB. *Escherichia coli* HypB belongs to the G3E family of P-loop GTPases that also includes UreG and MeaB,[88] which are accessory proteins involved in the production of urease and B12-dependent methylmalonyl-CoA mutase, respectively.[89, 90] There is considerable

variation between HypB homologs but all have NTPase activity and most have at least one metal-binding site.

NTPase activity of HypB is required for [NiFe]-hydrogenase maturation,[63, 80, 91] and although the purpose of this activity is not clear, it is likely to regulate HypB conformation and thereby facilitate selective nickel delivery.[79, 86, 92] In support of this model, the nucleotide-loaded state of HypB impacts nickel transfer from the protein in the presence of the other nickel accessory proteins.[86, 93] HypB homologs can be divided into two groups based on nucleoside-triphosphatase activity, either GTPase or ATPase. The *in vitro* GTPase turnover rate is quite slow, with hydrolysis on the order of one GTP per minute.[92, 94] The ATPase activity of HypB from *T. kodakaraensis* is accelerated in the presence of HypA,[79] but this is not the case with the *E. coli* proteins. The activity of *E. coli* HypB (*Ec*HypB) is not changed substantially in the presence of HybF[72] or HypA (C.D. Douglas, D.B.Z., unpublished observations), and is accelerated only several fold by SlyD,[93] suggesting that it requires the presence of a yet to be identified activation factor. The structures of HypB from *T. kodakarensis*, *H. pylori*, and *Methanocaldococcus jannaschii* have been solved by x-ray crystallography and all three reveal a homodimer with the nucleotide-binding site near the interface (Figure 5).[65, 92, 95, 96] Nucleotide and metal promote HypB homodimer formation, which has been observed in solution with micromolar dissociation constants.[95, 97-99] The generation of a HypB mutant unable to form dimers *in vitro* reduces hydrogenase production in bacteria but does not abolish it entirely, so the specific importance of the HypB dimer in [NiFe]-hydrogenase biosynthesis is still not clear.[96, 97]

Another variable in HypB proteins is the number and location of metal-binding sites. For example, species such as *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* encode HypB variants bearing a polyhistidine stretch (up to 24 histidine residues within a 39 amino acid

extension), capable of binding multiple nickel ions and contributing to nickel storage.[91, 100-102] However, many HypB homologs such as *Ec*HypB and *Hp*HypB do not have a His-rich sequence. The explanation for the significant variation in the metal-binding activities among HypB proteins is still not clear, but it may reflect the distinct nickel requirements of organisms that live in very dissimilar environments, or complementary functions in the other metalloproteins that contribute to [NiFe]-hydrogenase biosynthesis. For example, in *E. coli*, SlyD contributes to nickel storage,[93] and in *H. pylori* there are additional accessory proteins that play this role.[12]

*Ec*HypB has two distinct metal-binding sites.[103, 104] Spectroscopic analysis suggests that there may be communication between the two metal sites of *Ec*HypB because binding of one metal promotes subtle changes in the coordination of the other.[104] Furthermore, the presence of the two very different nickel-binding sites on *Ec*HypB, both required for [NiFe]-hydrogenase activation, suggests that there may be more than one pathway for nickel delivery to the hydrogenase enzymes in this organism, as discussed below.

The first type of metal site appears to be conserved in all of the GTPase HypB proteins,[87] but is not found in the archaeal ATPase variants.[79] This site on *Ec*HypB is often referred to as the 'low-affinity site' because nickel binds with a K_D on the order of 10^{-5} M and zinc binds 10-fold more tightly,[103] although the metal affinities of the corresponding site on *Hp*HypB are stronger by several orders of magnitude.[98] It is also referred to as the "G-domain site" because two cysteines and one histidine in the GTPase-domain were identified as ligands of this site.[95, 98, 103] Mutagenesis of these ligands revealed that this metal site on HypB is required for [NiFe]-hydrogenase biosynthesis in *E. coli*.[104] When this site is loaded with metal it decreases GTP hydrolysis,[92, 97, 99] providing a link between the two biochemical activities of the protein. This effect can be traced to the fact that one of the cysteine ligands is provided by the Switch II motif,

which connects to the nucleotide-binding site and makes functional conformational changes within the GTPase cycle.[92, 95, 105]

Crystal structures of *M. jannaschii* HypB and *Hp*HypB reveal either zinc or nickel loaded in the G-domain site, respectively.[92, 95] In both cases the metals bridge the protein dimer, but they possess distinct coordination.[92, 95] The *M. jannaschii* structure has two zinc ions in an asymmetric site comprised of two cysteines from each monomer but only one histidine, whereas *Hp*HypB contains a single nickel atom coordinated by four cysteines, two from each monomer, with the corresponding histidines disengaged from the primary coordination sphere (Figure 5).[92, 95] The difference in stoichiometry between the two metals was confirmed in solution and competition experiments indicate that nickel is bound an order of magnitude tighter in the presence of a non-hydrolyzable analogue of GTP compared to GDP, a trend not observed with zinc.[92] These biochemical differences hint that the distinct coordination environments may provide a means for the HypB G-domain metal-binding site to contribute to metal selectivity.

Some HypB proteins, such as *Ec*HypB, can also bind nickel at the N-terminus in what is referred to as the 'high-affinity site' because of sub-picomolar affinity.[103] Nickel is ligated in a square planar geometry with a CxxCGC motif via the three cysteine sulfurs and the primary terminal nitrogen (Figure 5),[104, 106, 107] and mutation of the cysteines completely disrupts nickel binding to this site *in vitro* as well as hydrogenase production *in vivo*.[104] The nickel coordination is maintained if the sequence is extracted in a short peptide,[107] and it also binds other first row transition metals according to the Irving-Williams series.[106] Given the tight nickel affinity of this site, if the nickel bound to the N-terminus of HypB is to be subsequently used in the pathway there must be some trigger that speeds up nickel release, and *in vitro* evidence suggests that this trigger includes SlyD.[93, 108]

SlyD. SlyD is a versatile member of the FKBP superfamily of PPIases (peptidyl-prolyl isomerases) that can also function as a protein folding chaperone and metallochaperone, with each activity delegated to a separate domain of the protein (Figure 6).[109] Unlike the complete abrogation of hydrogenase activity in *hypA/hybF* and *hypB* knock-outs, deletion of *slyD* in *E. coli* only reduces hydrogenase activity 2-fold to 10-fold, suggesting that it serves to optimize the efficiency of metal delivery.[59, 64, 108] The metallochaperone function is attributed to a C-terminal sequence with an unusually high density of metal-binding residues, including 15 histidines, 6 cysteines, as well as 11 aspartates and glutamates. This region is disordered in the absence of metal,[109] but is capable of binding multiple nickel ions in a mixture of coordination sites[110, 111] and is presumably the reason that SlyD can contribute to nickel storage in *E. coli*.[93] This protein also binds a variety of other transition metals with affinities that mirror the Irving-Williams series,[112] but there is no evidence that it contributes to the homeostasis of any metal besides nickel. Furthermore, SlyD does not confer metal resistance to *E. coli*,[112] suggesting a specific role in [NiFe]-hydrogenase maturation. Metal binding to the C-terminal domain of SlyD affects the PPIase activity [110] by modulating the structure of the PPIase domain.[113, 114] However, the PPIase activity is dispensable for its role as a hydrogenase maturation factor,[115] so this connection may implicate a regulatory role for metal on the other cellular activities of SlyD.

Protein Interactions and Metal Transfer. Several studies have established interactions between the individual hydrogenase nickel metallochaperones and have examined metal transfer between the proteins in efforts to outline the path that the nickel ion takes as it moves towards the [NiFe]-hydrogenase active site. Although how nickel is inserted into the *E. coli* hydrogenase precursor

proteins is not fully understood, it appears that there are at least two feasible scenarios stemming from the two nickel sites of *Ec*HypB (Figure 7). Whether both pathways are operational, or if each one is only functional under discrete conditions, remains to be resolved.

In one possible situation, the nickel from the N-terminal high-affinity site of HypB is ultimately delivered to the hydrogenase in a process facilitated by SlyD. SlyD was originally identified as a component of [NiFe]-hydrogenase biosynthesis when it was pulled out with tagged HypB proteins from *E. coli*[59] and *H. pylori*.[116] This interaction was mapped to surface residues of the *E. coli* SlyD IF domain,[108, 117, 118] which is responsible for the protein folding activity of SlyD.[119, 120] On the other side, SlyD interacts with a proline-rich region on HypB in between the GTPase domain and the N-terminal high-affinity nickel site.[108] SlyD also interacts directly with HycE through the IF domain, so this protein may contribute to hydrogenase production in multiple ways.[70]

SlyD can serve as a source of nickel for HypB *in vitro*[93, 118] but it also accelerates nickel transfer from the N-terminal high-affinity site of HypB to other acceptors.[93, 108] This latter activity of SlyD would provide a means to make the tightly bound nickel of HypB available in a biologically relevant timeframe, which is necessary if the nickel is to be employed. Complex formation between the two proteins, as well as the C-terminal metal-binding domain of SlyD, are required to accelerate nickel release from HypB, even though the nickel ion does not relocate to SlyD itself.[93, 108] The importance of this activity in hydrogenase biosynthesis is suggested by experiments with a SlyD mutant that has a partially-truncated metal-binding domain, which can still bind a nickel ion and form a complex with HypB but cannot fully activate nickel release from HypB. This mutant version of SlyD failed to complement a hydrogenase deficient Δ*slyD* strain of *E. coli*[117] thus indicating that stimulating nickel release from HypB is key to the role of

SlyD in [NiFe]-hydrogenase production. Furthermore, the activation of nickel release from HypB by SlyD is fastest when HypB is loaded with GDP,[93] raising the question of whether nickel release is triggered by GTP hydrolysis and if the GDP state is the functional form of HypB for nickel delivery.

In the second possible nickel pathway, nickel from the GTPase domain of HypB moves to HypA, which docks onto the large subunit and could enable nickel passage into the active site. HypB interacts with HypA in either a 1:1 or 2:2 complex (depending on the organism), as confirmed by numerous *in vitro* studies, pull down experiments, and GFP-fragment reassembly.[73, 79, 80, 99, 121] The complex can be suppressed by mutating several residues at the N-terminal side of the G-domain of HypB,[99, 121] leading to decreased hydrogenase production in *E. coli*.[86, 121] Examination of the *E. coli* proteins revealed that nickel rapidly moves from the low affinity G-domain site of HypB to HypA in a process that depends on complex formation between the two proteins and is not observed with zinc.[86] In addition, nickel transfer is further accelerated when HypB is loaded with GDP in a manner that is specific to HypA (versus a variety of small molecule chelators), suggesting that GDP primes HypB to rapidly and selectively transfer nickel to HypA. [86] Differences between the transfer of nickel and zinc from HypB to HypA could be a control point for achieving nickel-selective delivery to the hydrogenase, because even if HypB becomes loaded with zinc the metal would not be passed on to the next step in the pathway. However, the direction of transfer between HypA and HypB may be organism dependent, given the relative metal affinities measured for individual homologous proteins.[80, 98, 99]

Links to Nickel Homeostasis. Nickel as a nutrient can limit the amount of active hydrogenase.[122, 123] In *E. coli*, the nickel required for hydrogenase production is supplied by the Nik

transporter,[124, 125] which brings nickel through the periplasmic membrane as a complex with histidine.[126] How the nickel is then distributed to the biosynthetic pathway is still not defined, but there is some evidence that the nickel accessory proteins accumulate at the inner membrane,[58] suggesting that localization of the biosynthetic process, or even association with the transporter, is taking place to efficiently guide nickel to where it is needed. Production of the Nik transporter is regulated by the nickel-responsive metalloregulator NikR and is coupled to hydrogenase activity.[127, 128] In other organisms with higher nickel demand, such as *H. pylori*, the nickel homeostasis pathways are more complicated and include additional transporters and nickel accessory proteins.[12, 129] For example, the *H. pylori* GroES homolog HspA has an unusual C-terminal sequence that is rich in metal-binding residues, with 4 Cys and 8 His in the final 27 amino acids of the protein, and can bind several nickel ions per monomer.[130, 131] Modification of this sequence reduced hydrogenase activity as well as the cellular nickel content and nickel tolerance of *H. pylori*, but did not affect urease activity,[132] suggesting a dedicated role of HspA in hydrogenase biosynthesis analogous to that of *E. coli* SlyD. Two other small, His-rich *H. pylori* proteins Hpn and Hpn-like (Hpn2) can also bind multiple nickel ions and modulate nickel accumulation,[133-136] but a connection with hydrogenase production has not yet been observed.[137]

Proteolysis and Localization

Nickel-dependent proteolysis at the C-terminus is the final step in maturation of the LS and is believed to be a quality control checkpoint for the active site before it is assembled into the functional multiprotein complex.[138] Proteolysis is inhibited by transition metals other than nickel[139, 140] and is executed by a protease specific to each LS. The hydrogenase precursor protein is cut three residues downstream of the final cysteine in the primary sequence, which

serves as a nickel ligand.[141-143] How the proteases achieve specificity is still unclear but peptide swapping and mutagenesis experiments suggest that the sequences of the C-terminal peptides are not responsible for the selective activities of the proteases.[144, 145] Proteolysis of the LS may act as signal for association with the small subunit, followed by translocation through the TAT transporter, if required.[143, 145] Translocation of the LS across the membrane depends on the twin-arginine signal sequence found on the small subunit and association between the two subunits,[146, 147] and is aided by the HyaE and HyaF accessory proteins.[22, 148] There are examples of hydrogenases without a C-terminal extension, so how proteolysis contributes to subsequent stages of hydrogenase maturation, such as association with partners and/or localization, is still unclear. The association of the large and small subunits with other proteins permits hydrogenases to participate in specific biochemical pathways. In each case, variations in the hydrogenase large and small subunits change the electrochemical properties and tailor them to specific metabolic demands.[17] The ability for cells to take advantage of these chemistries is illustrated in *E. coli* where [NiFe]-hydrogenases used in hydrogen consumption are directed towards the periplasmic surface of the membrane whereas hydrogen producing [NiFe]-hydrogenases are coupled to the formate hydrogenlyase complex on the cytoplasmic face.[2, 8]

Future Steps

Based on all of these individual observations of the maturation proteins, we can assemble a complete view of the maturation of the [NiFe]-hydrogenase active site (Figure 8). While efforts to define this pathway have unveiled many intricate mechanisms behind the process, many details remain to be defined. Furthermore, it is clear that there are many variations between organisms,

particularly in the nickel delivery stage, and defining these differences will provide insight into how these organisms handle nickel homeostasis.

Some broader topics are also addressed by this research, with the potential to inform far-ranging cellular pathways beyond the production of the [NiFe]-hydrogenase enzymes. For example, there is the issue of the specificity of the accessory proteins. *E. coli* produces homologous versions of several of the accessory proteins, each one restricted to the maturation of a subset of the hydrogenase enzymes. It is not known why hydrogenase enzymes require some exclusive accessory proteins but others are pleiotropic, or how the protein specificity is mediated. Furthermore, there are reports of heterologous expression of hydrogenase structural genes, which rely on host maturation proteins to successfully produce active protein, suggesting that some systems are not as discriminating.[149-151]

Another question, common to the production of all metalloproteins, is how metal selectivity of metallocenter assembly is achieved. For example, there is clearly some mechanism of metal selectivity in the nickel delivery stage of hydrogenase maturation. Zinc inhibits nickel-dependent processing of the hydrogenase large subunit but only when the system is disrupted by introducing mutations that interrupt nickel uptake or in crude cell lysates, suggesting that the intact biosynthetic pathway provides a means to funnel nickel to the enzyme while excluding zinc.[139, 152] Defining how the correct metal moves between proteins and in the right direction is fundamental to understanding the mechanisms of metallochaperones. A growing body of work is implying that the fidelity of nickel insertion might not arise from characteristics of a metallochaperone that overcomes the Irving-Williams series, but instead stems from how these proteins interact and how metal is transferred from one protein to another. Future work will reveal how cofactors or partner proteins induce conformational changes, whether they are

accompanied by an impact on the coordinated metals, and how these adjustments lead to specific metal handover.

Figures

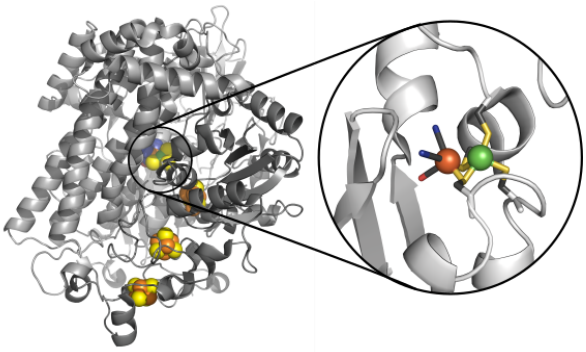

Figure 1. X-ray crystal structure of *Escherichia coli* Hydrogenase I (PDB 4GD3). The [NiFe] active site in HyaB, the large subunit (light grey), is shown magnified. The active site is comprised of nickel coordinated by four cysteinyl thiols, two of which bridge to iron that has two cyanide ligands and one carbon monoxide ligand. HyaA, the small subunit (dark grey), contains three iron-sulfur clusters that shuttle electrons to and from the catalytic center. The HyaA C-terminus interacts with HyaC and is truncated in this representation for clarity. Iron and zinc are shown as orange and green spheres, respectively, and sulphur is in yellow, nitrogen is in blue and oxygen is in red.

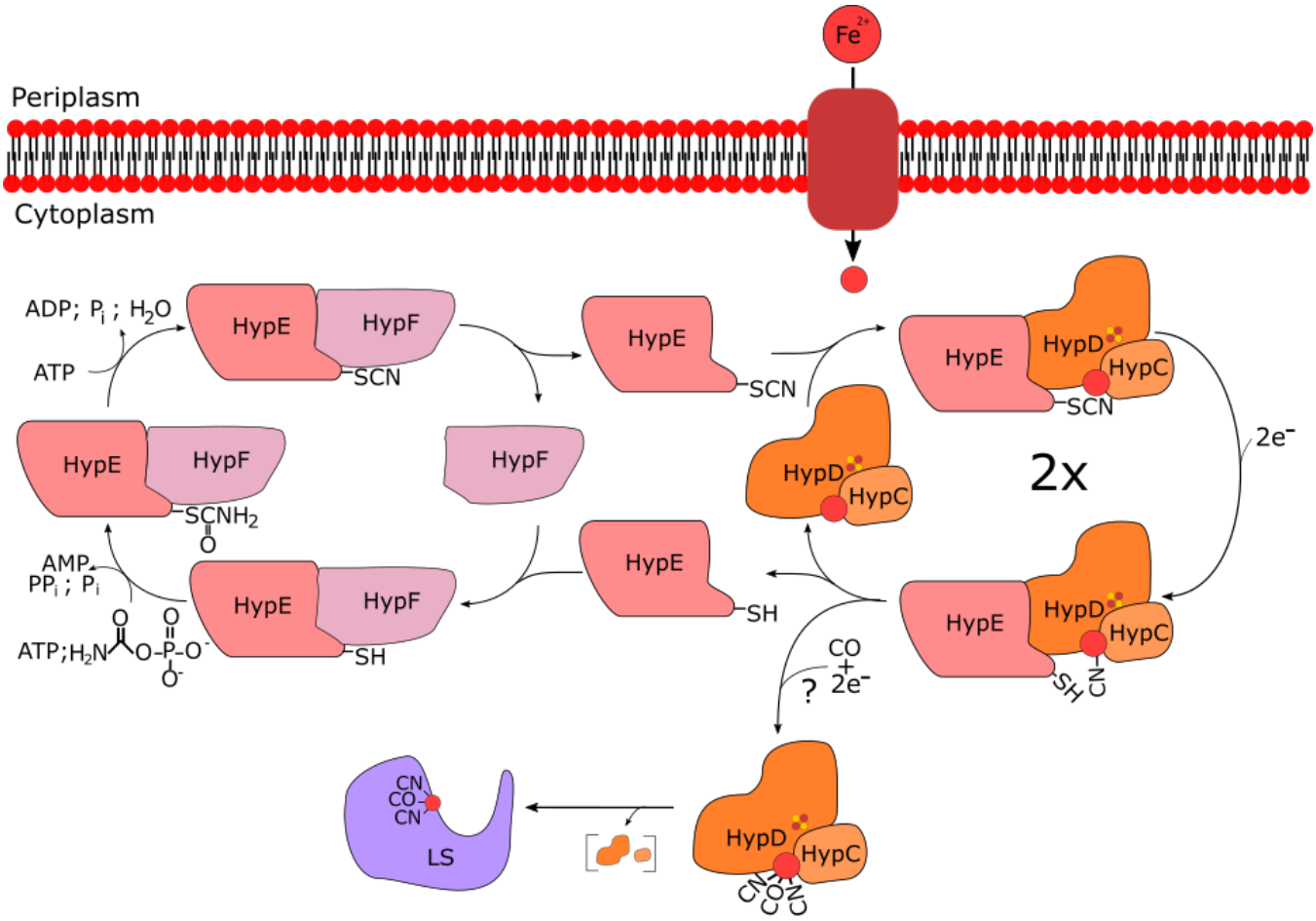

Figure 2. *Iron Center Assembly Scheme*: Iron is imported into the cell by a variety of possible transporters and associates with HypC and HypD, which together serve as an iron assembly scaffold. HypE and HypF generate cyanide ligands that are transferred to the iron center on the HypD-HypC scaffold. Carbon monoxide is then added through an unknown mechanism, and the iron center is subsequently delivered to the large subunit.

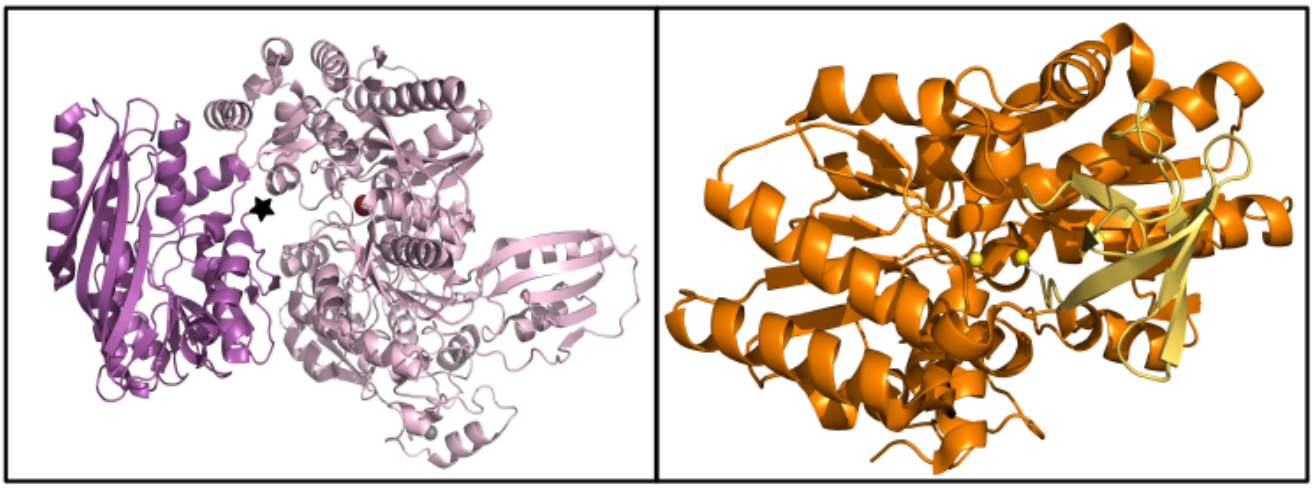

Figure 3. X-ray crystal structure of *Caldanaerobacter subterraneus* HypE-HypF complex (PDB 3VTI), left, and *Thermococcus kodakarensis* HypC-HypD complex (PDB 3VYS), right. **Left**: HypF (light purple) is a carbamoyl phosphate-dependent ATPase that transfers the carboamide moiety to the HypE (dark purple) C-terminus, indicated by a star, which is situated near the complex interface. **Right**: HypC (yellow) and HypD (orange) serve as a scaffold for the addition of carbon monoxide and cyanide onto iron. Two cysteinyl sulfurs, one from each subunit, indicated by spheres, act as iron ligands and serve as a scaffold where cyano and carbamyl ligands are assembled onto the iron.

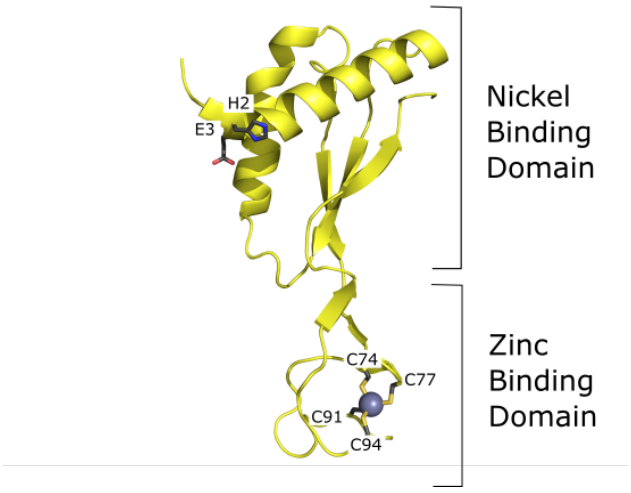

Figure 4. NMR structure of *Helicobacter pylori* HypA (PDB 2KDX). The two metal-binding domains are labeled as well as the metal-binding residues according to the wild-type sequence. The zinc domain is largely unstructured and has a tetrahedral tetrathiolate zinc site. The N-terminal residues His2 and Glu3, identified as vital for nickel coordination, are noted.

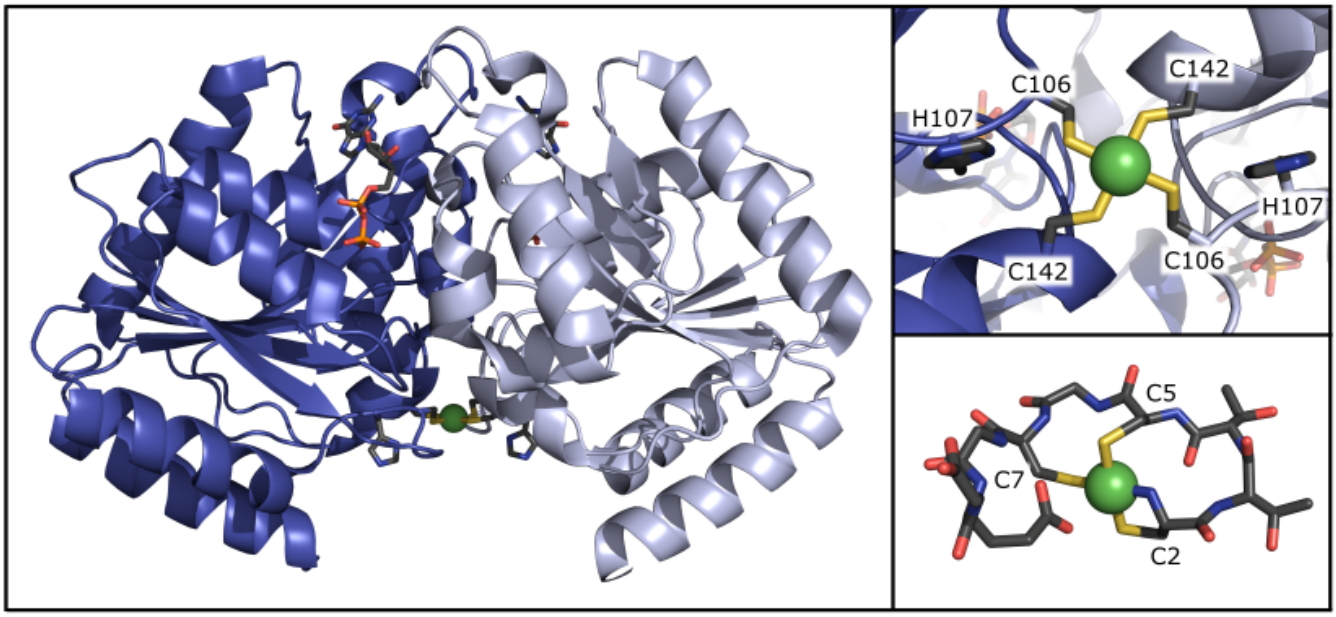

Figure 5. X-ray crystal structure of *Helicobacter pylori* HypB (PDB: 4LPS) and high-affinity site of *Escherichia coli* HypB. *Hp*HypB is shown as a homodimer, left, containing two molecules of GDP and a bridging nickel ion. Nickel is ligated in a square planar tetrathiolate site, top right, which connects the two HypB monomers and is located on an exposed surface. A DFT model of the *Ec*HypB high-affinity site[107], located at the N-terminus, is comprised of nickel coordinated with three cysteinyl sulfurs and the terminal nitrogen in a square planar geometry, bottom right.

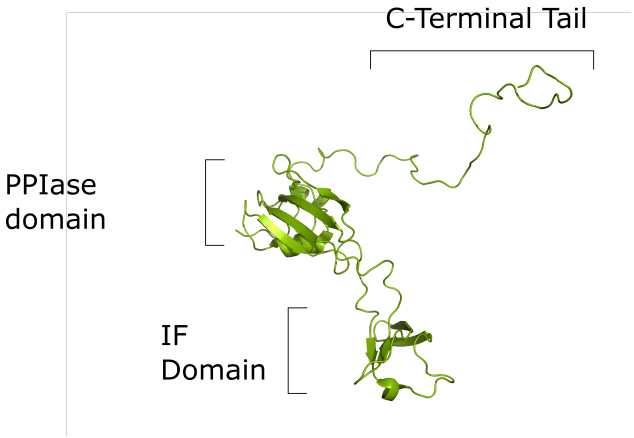

Figure 6. NMR structure of *Escherichia coli* SlyD (PDB 2KFW). The structured regions of the protein consist of the PPIase domain (top) and IF protein-folding domain (bottom). The C-terminal tail, comprised mostly of metal-binding residues such as His, Glu, Asp and Cys, is shown in a flexible extended conformation.

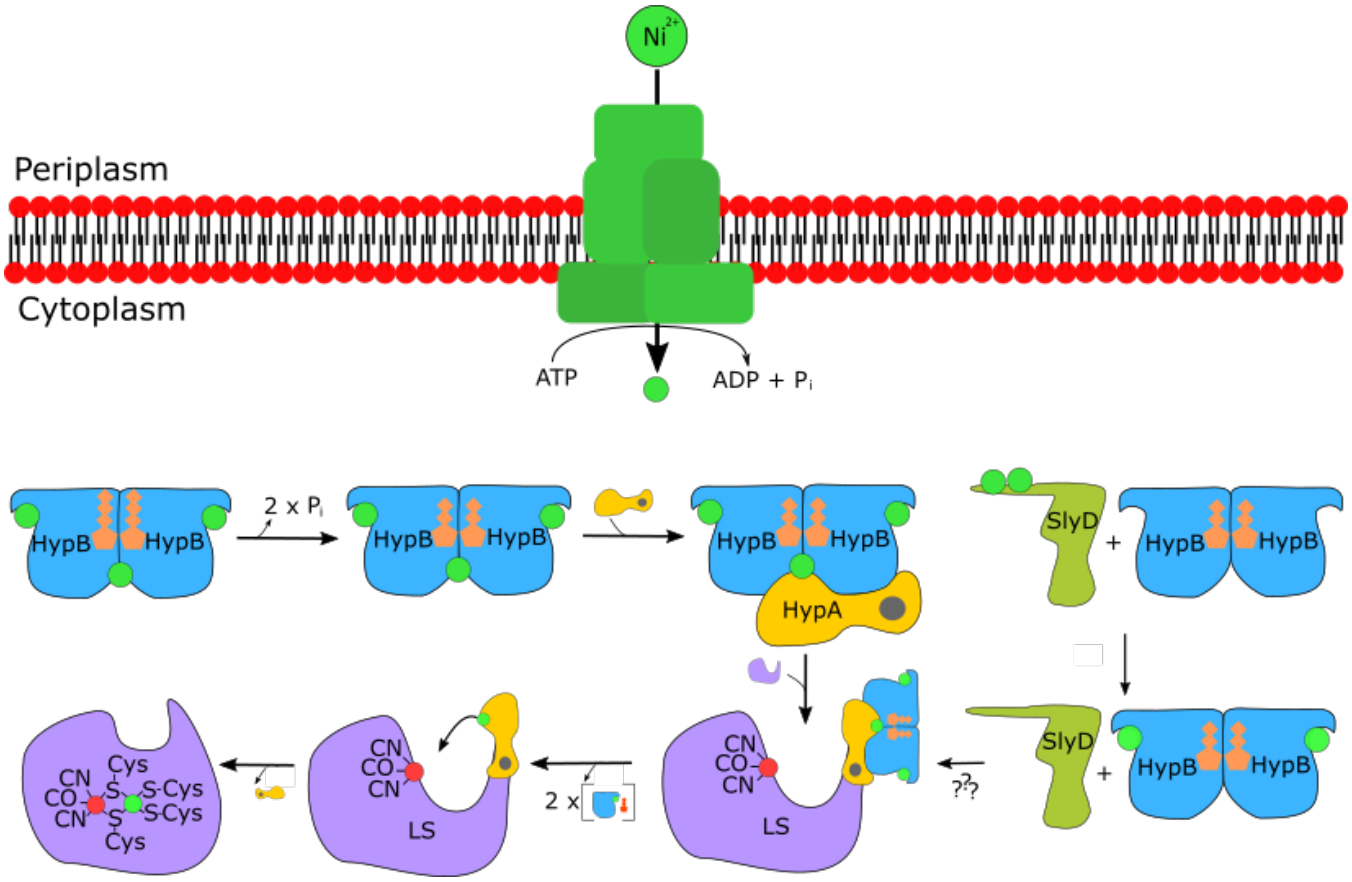

Figure 7. *Nickel Insertion Scheme:* Nickel is imported by the periplasmic ABC transporter NikABCDE. Once inside the cytosol, nickel is ligated by the high-affinity sites of HypB and SlyD. Nickel moves between SlyD and the HypB high-affinity site, and SlyD can activate nickel release from the HypB high-affinity site, an activity enhanced when HypB is loaded with GDP. Nickel can also bind to the HypB G-domain, and GTP hydrolysis results in a reduction of metal affinity and selective nickel transfer to HypA. HypA docks onto the primed large subunit where it can transfer nickel into the enzyme active site.

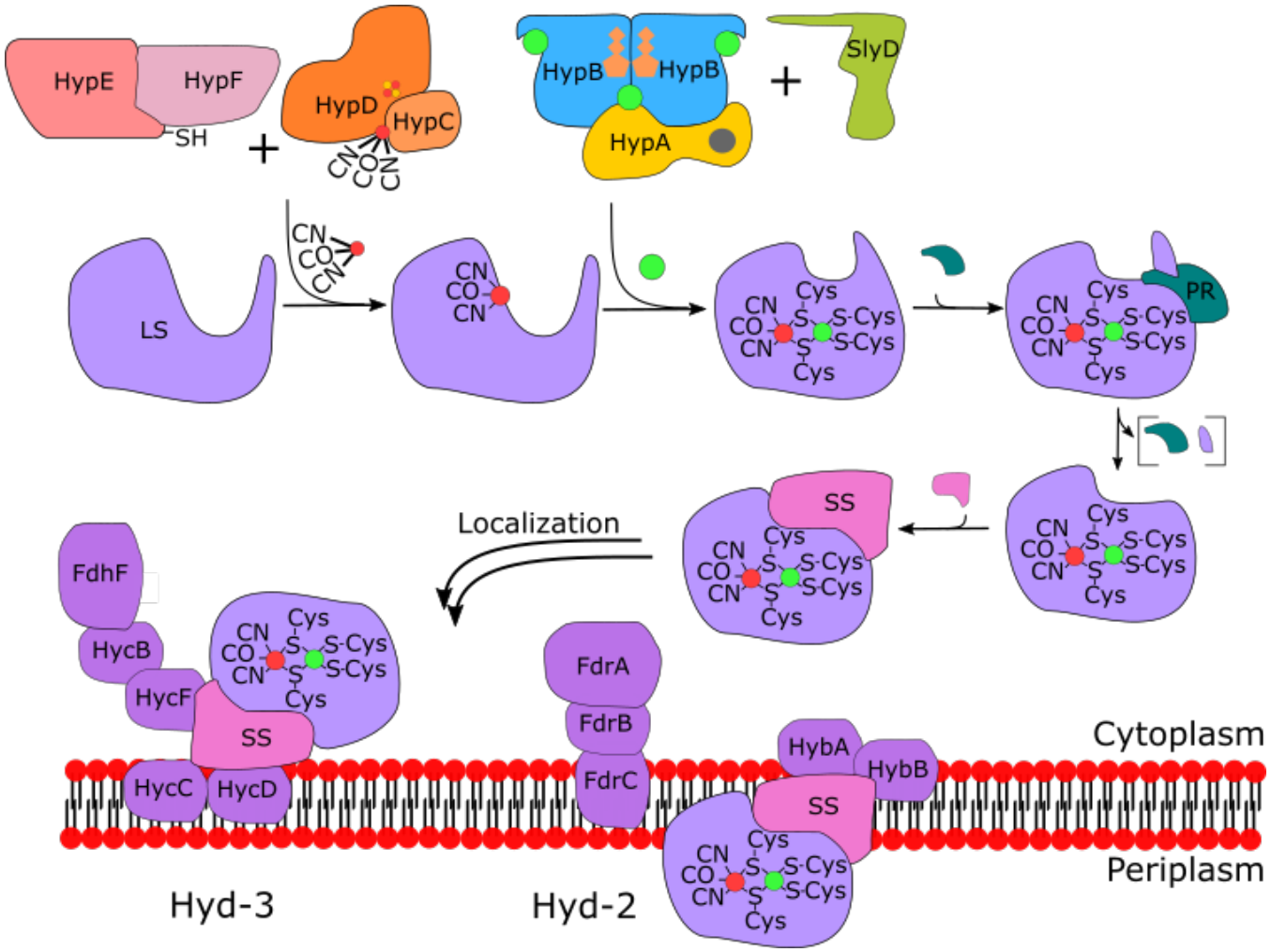

Figure 8. *Scheme of Escherichia coli [NiFe]-hydrogenase production*: Iron center assembly and delivery by HypC, HypD, HypE, and HypF is followed by nickel insertion by HypA, HypB, and SlyD into the large subunit (LS). A specific protease (PR) recognizes the completed active site and cleaves the C-terminus of the large subunit. The processed large subunit internalizes the active site via a conformational change, associates with the small subunit (SS), and translocates to the appropriate location. Hydrogenase-2 is transported across the membrane via the twin-arginine transport (TAT) system where is consumes hydrogen and supplies electrons via quinine pool to fumarate reductase.[2, 153] On the other hand, Hydrogenase-3 faces the cytoplasmic side as a vital component of the formate hydrogenlyase complex, and generates molecular hydrogen as a terminal electron acceptor.[8]

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