Nickel Homeostasis and Nickel Regulation: An Overview

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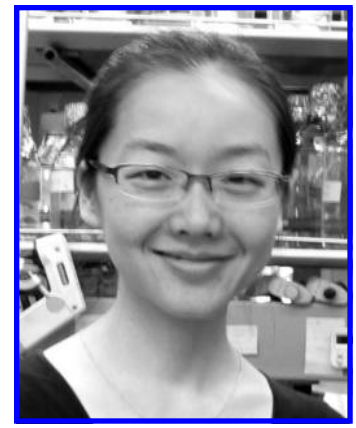

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1. Introduction

Nickel is an essential catalytic cofactor of enzymes found in eubacteria, archaebacteria, fungi, and plants.[1−6] These enzymes catalyze a diverse array of reactions that include both redox and nonredox chemistries[7] and allow organisms to inhabit a diverse range of environmental niches. For example, nickel proteins are key factors in the one-carbon metabolism of methanotrophs and methanogens, thus playing a significant role in the global carbon cycle.[4,8,9] In selected eukaryotic systems, nickel is required for the activity of the

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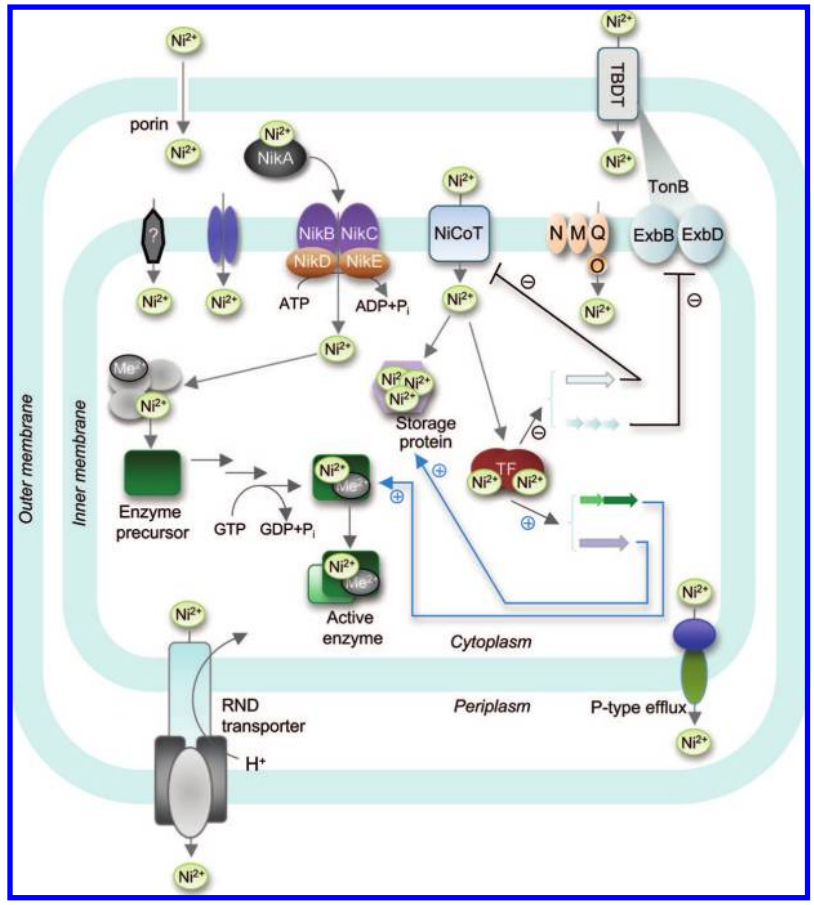

Figure 1. Schematic of different types of systems involved in nickel homeostasis. Some outstanding questions include how the nickel ions are transferred between the membrane transporters and the cellular nickel proteins, as well as the type of nickel complexes that are available in the extracellular milieu and periplasmic space. TF indicates transcription factor, and blue and black arrows indicate upregulation and downregulation, respectively.

urease enzyme, whereas the rest of the nickel enzymes characterized to date are confined to microorganisms, although some of these exist in symbiosis with higher organisms. As with other transition metals,[10,11] the use of nickel ions is inherently complicated because they are also potentially toxic and can cause a plethora of cellular damage,[12−14] so distribution and accumulation must be tightly controlled. Thus, in addition to the enzymes that employ nickel ions as chemical prosthetic groups, organisms with a nutritional requirement for nickel also express a variety of proteins that specifically contribute to nickel homeostasis by assembling metallocenters, importing or exporting the metal, transporting nickel ions within the cell, or regulating the expression of the factors involved. This micronutrient is ubiquitous in the environment, although the natural levels are usually quite low.[3,15,16] However, with the exception of nickel-hyperaccumulating plants and possibly marine plankton,[17−19] there is very little evidence of nickel deficiency in any organism that employs this metal, indicating that the uptake, trafficking, and storage mechanisms are efficient and sufficient. In this report, we attempt to summarize our current understanding of the pathways that contribute to nickel homeostasis and the mechanisms by which they are regulated (Figure 1). The structures of the known nickel enzymes are also briefly discussed in order to provide a comprehensive picture of intracellular nickel chemistry, but mechanistic details of the reactions catalyzed are not covered.

2. Transporters

2.1. Nickel Uptake

For nickel to be used in cellular pathways, the metal ions must be able to pass through the cytoplasmic membrane. It is apparent that low levels of nickel can travel through this membrane by way of multiple, possibly nonspecific, systems. However, in many of the microorganisms that require this metal dedicated nickel uptake transporters have been identified that belong to several different classes. Two examples that are primarily responsible for ushering nickel through the cytoplasmic membrane are the NikABCDE import pumps and the nickel/cobalt permeases (NiCoT).[2,20,21]

2.1.1. NikABCDE

NikABCDE belongs to the family of ATP-binding cassette (ABC) transporters that couple the translocation of a substrate across a cellular membrane to the hydrolysis of ATP.[22,23] Although the compounds handled by the members of this superfamily include a diverse array of molecules and metal ions, each system is fairly specific for its designated substrate. Sequence analysis and phylogenetic classification suggest that the Nik proteins are more closely related to oligopeptide transporters than to those that transport other types of metal ions.[24−26] On the basis of homology, the genes of the *nik* operon were designated as components of the expected core

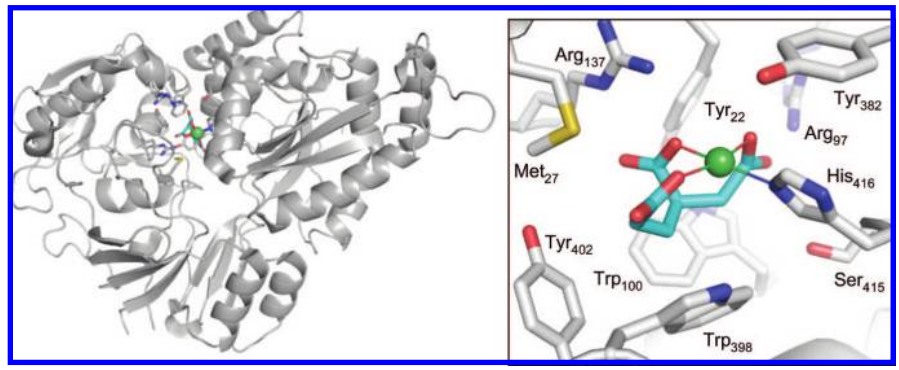

Figure 2. Structure of the NikA periplasmic-binding protein from *E. coli* (pdb 3DP8). The protein purified from the periplasm in the absence of EDTA contains a nickel bound to His416 and an organic ligand that is best modeled as butane-1,2,4-tricarboxylate (cyan) in a pocket between two protein lobes. The protein backbone is represented as a ribbon, and the residues surrounding the binding site are shown in sticks. The color scheme is nickel, green; oxygen, red; sulfur, yellow; and nitrogen, blue. The pictures were generated by using MacPymol.

structure: two channel-forming transmembrane proteins, NikB and NikC, and two cytoplasmic nucleotide-binding proteins, NikD and NikE.[24] NikA is a soluble periplasmic-binding protein (PBP) that acts as a receptor for nickel and presumably delivers it to the NikBC pore.[24,27] Originally identified in *Escherichia coli* as the source of nickel for the [NiFe]-hydrogenase enzymes expressed under anaerobic conditions,[28−30] Nik homologues have subsequently been found in several ureolytic pathogens, such as *Brucella suis*,[31] *Vibrio parahemolyticus*,[32] and *Yersinia* species,[33] and annotated in additional genomes.[25,34] In addition to activation during anaerobic growth (see section 4.1.1), NikA is induced in *E. coli* by exposure to mildly acidic conditions (pH 5.5) or upon biofilm formation,[35,36] and this latter effect is likely due to oxygen deprivation.[35]

Given that NikA is responsible for capturing nickel ions and delivering them to the membrane components for import into the cell, it represents a key factor in the nickel homeostasis of organisms utilizing this type of uptake mechanism. Intrinsic fluorescence experiments with *E. coli* NikA revealed quantitative nickel binding and afforded an upper limit of 0.1 μM for the K_D,[27] whereas binding by other metals was more than an order of magnitude weaker for Cu(II), Co(II), and Fe(II), or undetectable for Zn(II). In addition, characterization of the metal environment via X-ray absorption spectroscopy (XAS) suggested that the nickel was located in a site with six oxygen donors and a longer-range methionine ligand.[37] However, it is now unclear whether the experiments with aqueous metal salts are physiologically relevant because structural studies demonstrated that metal ions bind to NikA in a ternary complex with a small-molecule ligand.

The first high-resolution structure of NikA revealed a typical PBP conformation, with the substrate-binding pocket in a cleft between two globular domains that rotate with respect to each other about a flexible linker.[38] Although nickel binding causes some hinge closing in comparison to the apoprotein, the metal-binding site remains solvent-accessible. Furthermore, the binding pocket is composed of aromatic residues as well as arginines and methionine, an environment inconsistent with a role for NikA as a nickel-selective filter. The first coordination shell around the metal ion was tentatively modeled to include five water molecules with unusually long Ni−O distances as well as a cation-π interaction with tryptophan. In a second study, the protein was resolved with an Fe(III)−EDTA$(H_2O)^-$ complex bound at the same location.[39] Ethylenediaminetetraacetic acid (EDTA) was included during the purification of the aerobically expressed, recombinant protein, and although iron was not deliberately added, it would have been present in trace amounts. The Fe(III)−EDTA complex remained bound to NikA after several chromatography steps and even upon growing crystals in the presence of $NiCl_2$,[39] indicating a tightly bound ternary complex. Furthermore, a metal ion bound to NikA in the context of a carboxylate-containing chelator is reasonable given the chemical composition of the substrate-binding pocket, and interactions between two arginine residues and the EDTA were noted. These experiments suggest that NikA binds a nickel−metallophore that is translocated across the periplasmic membrane, analogous to the Fe(III)−siderophore uptake mechanisms. The affinity of NikA for Ni(II)−EDTA is only 30 μM,[40] but it is not likely to be the physiological complex. In an attempt to identify the natural metallophore, holo-NikA was purified from the *E. coli* periplasm in the absence of EDTA (Figure 2).[41] In the resulting structure, the compound bound to the metal was best modeled as butane-1,2,4-tricarboxylate (BTC), which contributes three carboxylate ligands to the square-planar metal coordination site. The fourth ligand is a histidine residue, and the two protein lobes are significantly more closed around the metal.[41] However, the Ni(II)−BTC−NikA complex could not be reconstituted from apoprotein, and the authors pointed out that there is no obvious metabolic route for BTC biosynthesis in *E. coli*, so the identity of the metallophore required for anaerobic nickel uptake remains to be defined.

A crystal structure of NikA with many of the metal-pocket residues mutated revealed a second nickel site on the surface of the protein with nickel coordinated by two histidines and four water molecules.[40] Calorimetry was used with this mutant to measure a 10 μM affinity for aqueous nickel,[38,40] and the authors pointed out that this site most closely matches the initial XAS data.[37] The location and the lack of conservation of the histidine residues suggest that this site is not critical for nickel transport, but it may be a component of NikA's role in the negative chemotaxis response of *E. coli* to high concentrations of nickel.[27,40] Along the same lines, it would be interesting to determine whether NikA is critical for the promotion of *E. coli* biofilms observed with subinhibitory concentrations of nickel.[42]

It was recently demonstrated that NikA can bind heme moieties with 0.5 μM affinities that are independent of nickel, suggesting a potential role for NikA in heme processing.[43] The dipeptide-binding PBP DppA, a NikA homologue, also

binds heme and contributes to the use of heme as a source of iron, but NikA does not influence this pathway.[44] A possible link between nickel and heme was noted because the activity of [NiFe]-hydrogenase enzymes may be coupled to cytochromes,[43] but more information about the role of this protein in heme metabolism and how this activity influences nickel homeostasis is necessary.

2.1.2. NiCoT

The NiCoT family members are secondary transporters found in many gram-positive and gram-negative bacteria as well as several archaea and fungi.[21] The high-affinity nickel permeases include HoxN of *Cupriavidus necator* H16 (previously named *Alcaligenes eutrophus* and *Ralstonia eutropha*)[21,45] and NixA from *Helicobacter pylori*.[46] These monomeric,[47] single-component permeases have a K_M for nickel of ~10−20 nM but low transport capacity.[46,48−50] This family is divided into subgroups with varying degrees of substrate preferences for nickel versus cobalt,[21] which for many has been defined by examining metal transport in a heterologous host. HoxN is selective for nickel, and transport is not inhibited by 10-fold excess of Co(II).[50,51] Other family members transport both nickel and cobalt but have a preference for one metal over the other,[50−52] and it has been noted that the substrate specificity of these transporters sometimes correlates with whether the gene colocalizes with nickel- or cobalt-utilizing systems.[50,53] With respect to other metals, nickel transport by HoxN and other systems is not inhibited by a 10-fold excess of Zn(II) or Mn(II).[50,51] However, nickel uptake by NixA and cobalt uptake by NhlF from *Rhodococcus rhodochrous J1* is significantly reduced in the presence of 100-fold excess Zn(II),[51,54] so the selectivity of transport may depend on both the absolute and relative metal concentrations. Furthermore, it is not yet clear if transport is hindered by other metals such as zinc because they are competitive substrates or just inhibitors. The observation that HoxN only causes a 10-fold increase in intracellular nickel concentrations when expressed in *E. coli* suggested a slow, uniport mechanism of transport.[49,51] It is possible that these proteins are driven by the proton gradient across the cytoplasmic membrane because uncouplers decrease cobalt transport by NhlF,[52] but this connection has not been shown for any other system.

A topological model of these integral membrane proteins has eight transmembrane helices, proposed on the basis of hydropathic index and the cellular localization of a series of fusion proteins.[47,55,56] Mutagenesis of a highly conserved His-Xaa_4-Asp-His motif, which is imbedded in the second putative transmembrane segment and is also found in *E. coli* NikC,[49] reduced transport and the ability to compete for the metal ions with other media components.[54,56,57] The importance of the NiCoT signature sequence was highlighted by the demonstration that a phenylalanine in the third position correlates with higher capacity but lower selectivity for nickel versus cobalt, whereas a valine produces the opposite effect.[57] An examination of additional mutants and chimeras led to the prediction that transmembrane domain I contributes to metal selectivity in concert with domain II,[57] as do conserved residues in other transmembrane segments,[54,57] including a GlyHisSer motif in the third transmembrane helix.[21,54] In contrast, mutagenesis of predicted periplasmic and cytoplasmic loops or of the cysteines distributed throughout the protein did not have significant effects on nickel transport.[54,56]

UreJ/HupE and UreH are related permeases found in the hydrogenase or urease gene clusters of many bacteria,[21,33,58−61] and they seem to be relatively specific for nickel.[21] These transporters are predicted to have six transmembrane segments with the signature His-Xaa_{4-5}-Asp-His motif located in the first one. Variants of the nickel permeases have also been predicted in cyanobacteria, encoded by genes associated with that of Ni-SOD, and plants,[21] and a NiCoT homologue is required for nickel transport and urease activity in fission yeast.[62]

2.1.3. Additional Periplasmic Transporters

In the case of many of the membrane transporters mentioned above, interruption of the nickel uptake activity does not completely block nickel accumulation and/or the activity of the nickel-dependent enzymes, suggesting that there are redundant routes for nickel influx. For example, disruption of the *H. pylori nixA* gene only reduces the urease activity in some strains by about 50%,[63,64] although this is sufficient to affect both the level and distribution of gastric colonization in a mouse model and results in a strain that cannot compete with wild-type *H. pylori* in vivo.[64] In another example, the hydrogenase deficiency of *E. coli nik* mutants is complemented by growth in media supplemented with micromolar nickel,[28,29] which is thought to enter the cell through the CorA Mg(II) transporter.[65] Furthermore, mutating both the Nik and CorA systems does not completely inhibit nickel sensitivity,[24] suggesting that there is at least one alternative means for nickel to enter *E. coli.* This hypothesis is further supported by the recent demonstration that nickel-responsive regulation of the *nik* promoter by the metalloregulator NikR is not influenced by the presence of the Nik proteins.[66]

A recent search of available prokaryotic genomes revealed many homologues to the known families of nickel and/or cobalt transporters.[53] These putative transporter genes were subsequently annotated for substrate specificity based on upstream regulatory elements or colocalization with genes for nickel- or cobalt-dependent enzymes.[53] The type of transporter most frequently found in this study was CbiMNQO, which has been identified near the urease operons of *Actinobacillus pleuropneumoniae* and *Streptococcus salivarius* 57.I. and is important for urease activity if the medium is not supplemented with extra nickel.[67,68] This family includes several variants that always have an annotated cytoplasmic ATP-binding protein and transmembrane components,[53] and it was proposed that the transporters involved in nickel homeostasis be given the "Nik" designation instead of "Cbi".[53] Unlike the NikABCDE system discussed above, the gene for a soluble PBP homologue was not found with any of the CbiMNQO sequences and was not necessary for active metal uptake upon heterologous expression of several of the operons,[53] so the mechanism of metal influx may be different. This in silico analysis found at least one putative transporter in two-thirds of the microorganisms examined, with some species having redundant sets of genes.[53] The significant number of bacteria that use nickel without a homologue of the known nickel/cobalt transporters is more evidence that there are mechanisms of nickel uptake that have yet to be identified.

2.1.4. Outer Membrane Transporters

Cellular nutrients for gram-negative bacteria must first pass through an outer membrane prior to reaching the cytoplasmic membrane. Small molecules and ions can diffuse through nonspecific transmembrane porins in the outer membrane,[69,70] so it was assumed that nickel ions would get into the periplasm by this mechanism. However, there is now evidence that, at least in some organisms, nickel uptake across the outer membrane occurs through a TonB-dependent transporter (TBDT).[70,71] This type of pathway has high substrate affinity and specificity and is the means of uptake for a variety of iron complexes as well as vitamin B12.[70,72,73] A TBDT receptor is essentially a barrel spanning the outer membrane with a plug at the bottom, and the interaction with TonB on the periplasmic side couples the opening of the plug and substrate passage to the electrochemical charge gradient of the inner membrane. The *H. pylori* genome contains 6 TBDTs originally annotated as iron-complex receptors, but it turns out that only 4 of these genes display the expected iron-mediated expression.[74] The other two genes, FecA3 (HP1400) and FrpB4 (HP1512), are repressed by the nickel-responsive transcription factor HpNikR (section 4.1.2),[75−78] which is also responsible for nickel-dependent transcription of the TonB genes in this organism.[77,79] Knocking out one of these components reduces urease activity and nickel uptake,[76,79] particularly upon growth at acidic pH, suggesting that these receptors are involved in TonB-dependent transport of nickel. This surprising discovery implies that the imported nickel exists in the medium as a complex with one or more small-molecule ligands. The nature of the nickel complex(es) and whether it resembles the molecule captured by *E. coli* NikA is not yet clear. Furthermore, although putative nickel TBDTs have been assigned in several prokaryotes,[53,70] they have not yet been found in all of the gram-negative organisms that are known to employ nickel (e.g., *E. coli*), so the extent to which this mechanism is used and how it integrates with the inner membrane transport systems remain to be established.

2.2. Nickel Export

As with other metal ions, Ni(II) is toxic in excess.[12,13] Thus, even organisms that do not have a nutritional requirement for this metal may need a mechanism to deal with superfluous nickel, and the most common mechanism for metal resistance is metal efflux (reviewed in ref 80). Several exporters have been predicted and/or shown to pump nickel out of the cytoplasm[80] or even the periplasm,[81] but in only a few cases are the transporters specific for nickel. An example is NreB from *Achromobacter xylosoxidans* 31A, a putative exporter from the major facilitator superfamily with 12 transmembrane segments and a histidine-rich C-terminus.[80] NreB is specifically induced by nickel and confers nickel resistance and reduced nickel accumulation upon heterologous expression in *E. coli*.[82] Another example found in *Thlaspi goesingense*, a nickel-hyperaccumulating plant that sequesters nickel into the leaf vacuoles,[17,83] is the constitutively expressed member of the cation diffusion facilitator family of membrane transporters named TgMTP.[84] This protein provides resistance to metal-sensitive yeast and, depending on the splice variant, is specific for either Cd/Co/Zn or nickel.[84] It is expressed at much lower levels in related plant species that are not nickel-tolerant,[84] so it is likely that it contributes to the ability of *T. goesingense* to accumulate nickel, but whether or not it is responsible for vacuolar storage is not yet clear.[85]

The lack of nickel efflux systems identified in organisms that use this metal implied that the control of nickel accumulation is accomplished primarily via carefully regulated uptake.[80] However, nickel exporters have recently been found in both *H. pylori* and *E. coli*. In the first case, mutating the Hp0969-0971 genes prevents *H. pylori* colonization of gerbil stomachs and increases the sensitivity of the bacteria to cadmium, zinc, and nickel, but not to other metals, consistent with the hypothesis that they constitute a metal-specific efflux pump that was named CznABC.[86] This assignment was supported by *cznA* or *cznC* mutants that produced a dramatic increase in urease activity under standard growth conditions without affecting urease protein levels, suggesting that nickel availability was enhanced. Surprisingly, the *cznB* mutation did not have the same effect. CznABC is proposed to be a novel system that pumps the metal ions across both the inner and outer membranes, with some similarities to the CzcCBA resistance-nodulation-division (RND) transporter.[80] In *E. coli*, RcnA (previously YohM) is a membrane protein thought to be a nickel exporter because it contributes positively to nickel and cobalt resistance and negatively to nickel accumulation.[87] The gene for RcnA, which was picked out of the *E. coli* genome in a bioinformatics search for histidine-rich proteins,[87] is induced by both nickel and cobalt,[87−89] but not other metals,[87] although it may also provide a connection to iron homeostasis.[88] The proposed topology of RcnA includes 6 membrane-spanning segments, and there is only limited homology with other transporters families,[87] so it may be the prototype for a new class of efflux systems.

3. Nickel Enzymes: Structure and Assembly

There are multiple enzymes that employ nickel as a cofactor to catalyze a diverse array of chemical reactions. In several of these enzymes, the nickel is embedded in an intricate, multinuclear cluster that also includes modified amino acids and/or exogenous ligands. As with other complex metallocenters,[90] these nickel sites are assembled on protein scaffolds by teams of dedicated accessory proteins. Some of the helper proteins can be replaced by an unnatural excess of a chemical cofactor, reflecting the controlled distribution of these potential toxins that is normally maintained in a healthy cell. Although many of the details of the multistep pathways are not yet understood, in several cases the overall processes have been outlined and a number of the individual components are at least partially characterized. One common theme is the requirement for an NTP-hydrolyzing protein, but it is not yet clear how the energy is spent. Another common theme is that nickel metallochaperones have been identified that presumably deliver the metal ion to the apo-enzymes even under conditions of low intracellular availability. Some organisms also express factors capable of nickel storage that could keep the excess metal sequestered in order to prevent cellular damage and serve as a backup resource in situations of reduced environmental nickel. There are several nickel enzymes for which accessory proteins have not yet been identified, but given the tight supervision of this element in many systems, it is likely that there are factors responsible at least for the delivery of nickel to these enzymes.

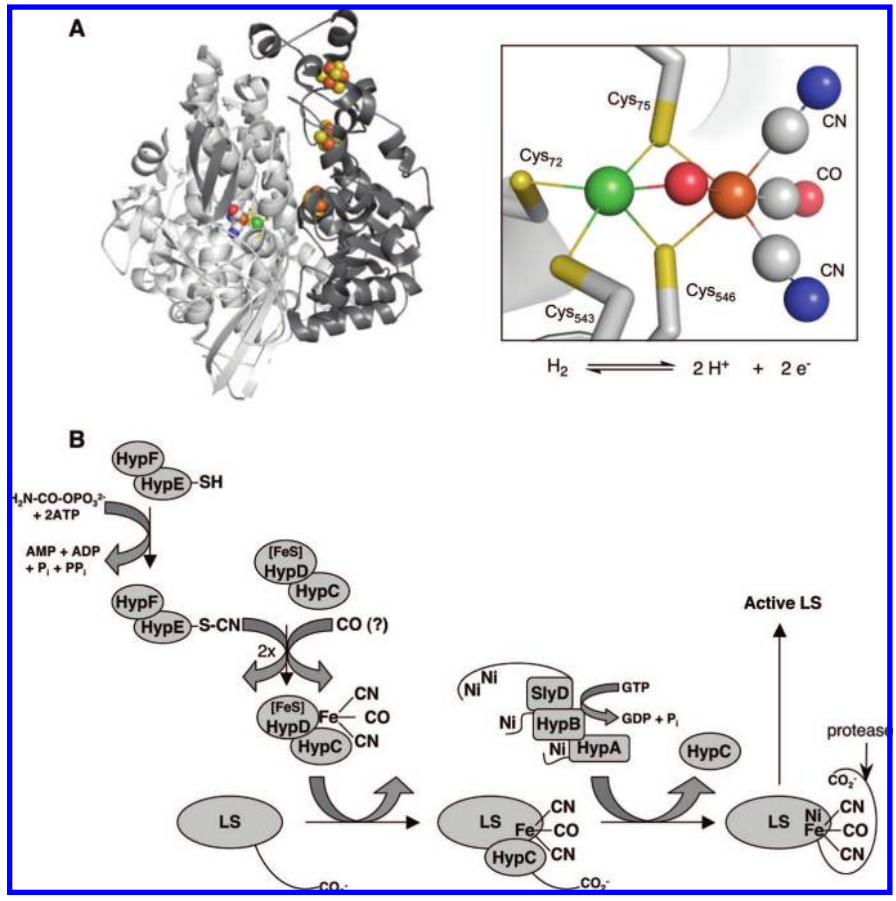

Figure 3. Structure and assembly of the [NiFe]-hydrogenase. (A) Structures of the ready oxidized form of the [NiFe]-hydrogenase from *Desulfovibrio fructosovorans* (left, pdb 1YRQ), and the [NiFe] active site (right). The presence of the bridging ligand and its identity depend on the state of the enzyme. The large subunit is depicted as a light gray ribbon, and the small subunit is in dark gray. The color scheme is nickel, green; iron, orange; oxygen, red; sulfur, yellow; and nitrogen, blue. The structures were generated by using MacPymol. (B) Working model for the assembly of the [NiFe] center on the large subunit (LS) of the [NiFe]-hydrogenase, based primarily on our understanding of the *E. coli* factors. HypC and HypA can be replaced by the homologous HybG and HybF, respectively, and SlyD is not found in all organisms. Assembly and association with the small subunit are not shown. Adapted with permission from ref 113. Copyright 2007 Elsevier.

3.1. [NiFe]-Hydrogenase

Hydrogenase enzymes catalyze the formation of hydrogen gas from protons and electrons and/or the reverse reaction. These enzymes are a central component of microbial energy metabolism in organisms that flourish in a diverse array of environmental conditions.[2,91−93] Hydrogenases can oxidize H_2(g) as a source of reducing power linked to energy-conserving pathways, produce it as a means of disposing excess reducing equivalents, or act as sensors for the availability of hydrogen gas. The [NiFe]-hydrogenases are a phylogenetically distinct class of hydrogenase enzymes that have both nickel and iron at the active site and are widely distributed in bacteria and archaea.[92,94,95] For example, a [NiFe]-hydrogenase is required for efficient colonization by *H. pylori*,[96] a human pathogen that causes peptic ulcers and some types of gastric cancers.[97,98] Another recent study of *Salmonella enterica* serovar Typhimurium, a common food poison, revealed that a mutant lacking hydrogenase activity was avirulent in a typhoid-fever mouse model in marked contrast to the lethal parent strain.[99] These infectious organisms use the hydrogen produced by other microorganisms in the body, a resource not used by the human host, as a supply of energy.[96,100−102]

The [NiFe] dinuclear active site is buried in the large subunit (LS) of a heterodimeric protein (Figure 3),[103] with the nickel ion bound to the cysteines of two CxxC motifs, one of which is located a few residues away from the C-terminus.[92] One cysteine from each pair bridges to the iron center that also has a carbon monoxide molecule and two cyanides serving as ligands, and in some organisms one of the nonbridging cysteine residues can be replaced by a selenocysteine.[92,104] It is likely that the iron center is assembled and inserted into the hydrogenase protein before nickel delivery.[105] In support of this model, the LS of *C. necator* was isolated fully loaded with iron but containing only substoichiometric amounts of nickel,[106] and spectroscopy revealed the expected diatomic ligands on the iron.[107] The biosynthetic genes in *E. coli*, which expresses at least 3 hydrogenase isoenzymes, were given the *hypA-F* designation because most of them contribute to the maturation of all of the active hydrogenase enzymes in a pleiotropic manner,[108−110] although in several cases there are alternative homologues that are only involved in the biogenesis of specific isoenzymes.[111,112]

Much of the current knowledge about the biosynthesis of the iron ligands and iron insertion, as well as the subsequent steps, is derived from the experimental work carried out by the group of Böck on the maturation of *E. coli* hydrogenases (Figure 3), which was recently reviewed.[90,103,104,113,114] Briefly, HypF forms a complex with HypE[115−118] and uses ATP to

drive the transfer of the carboxamide group of carbamoylphosphate to the C-terminal cysteine of HypE, where dehydration coupled to the hydrolysis of a second ATP produces a HypE-SCN complex.[115,119−121] HypE then interacts with a reduced HypC/HypD complex[116,117,122] to which it transfers the cyanide.[122] HypD contains an [4Fe−4S] cluster[122−124] that could serve as the source of the hydrogenase iron and/or reducing equivalents. The origin of the carbon monoxide ligand is not clear,[114] but the biosynthetic pathway may include acetate but not carbamoylphosphate.[125−127] Furthermore, whether the iron center is preassembled on a scaffold and then transferred intact to the LS or whether it is constructed piecemeal on the LS is not yet known, although there is evidence for the first model in at least one system.[128]

After delivery of the iron center, HypC or its homologue remains in a complex with the specific LS,[105,106,116,117,129] which is now primed for nickel insertion.[130] This complex requires the first cysteine in the LS sequence that serves as a terminal nickel ligand and the N-terminal cysteine of HypC,[111,131] which is also critical for interaction with HypD.[105] Given that reducing agents do not inhibit complex formation but alkylating agents do, it is feasible that the cysteine of HypC temporarily coordinates the iron.[104,131] Cys1 of isolated HypC is solvent-exposed and is surrounded by a cleft of conserved hydrophobic residues, whereas another part of the protein surface has an acidic patch and the C-terminal region appears to be flexible, all features that may be important for protein−protein interactions with several distinct partners.[124,132] A role for HypC as a folding chaperone that holds the LS in a conformation ready to accept the metal centers has been proposed, but additional responsibilities have not been ruled out.

Following nickel insertion, discussed in detail below, the HypC protein dissociates from the LS[130] and an isoenzyme-specific protease cleaves the C-terminal tail of the LS three residues downstream of the final cysteine ligand (reviewed in refs 104 and 133). Although there are a few examples of hydrogenase LSs that do not have a C-terminal extension,[104] if it is present it is thought to be an essential component for metallocenter assembly.[134] The crystal structure of *E. coli* HybD (the hydrogenase 2 protease) revealed a similar topology to the metzincins superfamily of zinc endoproteinases as well as a Cd(II) ion that was supplied from the crystallization buffer in a surface-exposed site.[135] The solution structure of HycI (hydrogenase 3 protease) in the absence of metal is similar but more "open" around the metal-binding site.[136] Given that the proteases will not cleave the LS until nickel has been incorporated into the LS[137−141] and mutation of the putative metal-binding residues of the protease inhibited activity,[140] it is feasible that the nickel ion in the assembled hydrogenase metal center serves as a signal that activates the protease and/or as a cofactor for hydrolysis.[104] However, the proteases are not inhibited by standard protease inhibitors besides divalent metal ions and chelating agents, and the mechanism of peptide bond hydrolysis is unknown. Once the C-terminal peptide is removed, there must be a conformational change that closes up the protein around the metal cluster, resulting in the mature form of the subunit. The next step is association with the small subunit (SS), which contains Fe−S clusters that participate in electron transfer to and from the active site[103] and presumably is prepared in parallel by an independent biosynthetic pathway.[104] Finally, the completed enzyme system is directed to its ultimate cellular destination. Many hydrogenases need to be transported intact to the outer side of the cytoplasmic membrane, a process that occurs via the twin-arginine translocation (TAT) system[142−144] with help from additional accessory proteins.[145,146]

3.1.1. HypB

The hydrogenase deficiency caused by disrupting *hypB*, *hypA*, or their homologues in several different organisms can be at least partially complemented by nickel supplementation of the growth media,[108,112,147−153] implicating these proteins in the nickel delivery step of hydrogenase biosynthesis. The observation that they can be functionally replaced by an excess of nickel suggests that they are not chemically necessary for hydrogenase biosynthesis. Instead, given that close-to-toxic levels of nickel are required in their absence and full complementation is not achieved,[104] it is likely that they guide the nickel to the precursor protein and/or stimulate the rate of incorporation.

The C-terminal domain of HypB is classified as a P-loop GTPase, and mutations in HypB that disrupt GTP hydrolysis in vitro also prevent nickel insertion and hydrogenase maturation in vivo.[147,155,156] The intrinsic activity of purified HypB is low, with a k_{cat} of 0.2 min^{-1} and a K_M of 4−7 μM measured for the *E. coli* and *Bradyrhizobium japonicum* proteins,[157,158] although the *H. pylori* HypB is slightly faster[159] and no activity was detected with *Rhizobium leguminosarum* HypB.[152] The protein is a dimer in solution[158,159] and the GTP-binding site bridges the two monomers,[160] so it is possible that a HypB dimer is the functional GTPase. However, given the feeble GTP hydrolysis of isolated HypB, it is likely that the activity is stimulated in the context of the nickel insertion complex. There are many models for how GTP binding and hydrolysis contribute to nickel delivery, including regulating protein−protein interactions to form the active complex, nickel release and/or transfer, or dissolution of the nickel-insertion complex following successful placement of the nickel. This outstanding question may also be relevant to the role of the NTPases employed in the biosynthesis of other nickel-containing enzymes discussed below.

Some HypB homologues have a histidine-rich region near the amino terminus that can bind multiple nickel ions and is thought to be involved in nickel storage.[149,152,157] HypB was also implicated in the transcription of the hydrogenase structural genes in *B. japonicum*,[149] but the nature of this relationship has not been defined. Deletion of the histidine stretch of residues from *B. japonicum* HypB produced a protein that could still bind at least one nickel ion in vitro and support hydrogenase biosynthesis in vivo,[149,156] suggesting that the two roles of nickel storage and nickel delivery can be separated. The modification to the *B. japonicum* protein did not disrupt the N-terminal CxxCGC motif that is conserved in many HypB proteins.[161] This sequence is responsible for a high-affinity nickel-binding site in *E. coli* HypB, which binds stoichiometric nickel with 0.1 pM affinity in a competition experiment.[162] XAS analysis of this complex, in conjunction with mutagenesis and chemical modification, demonstrated that the nickel is coordinated to the three cysteines as well as the nitrogen of the amino terminus in a square-planar geometry (Figure 4A).[162−164] Furthermore, mutagenesis of the three cysteines demonstrated that this site is essential for hydrogenase biosynthesis in *E. coli*.[164] Although the 3-dimensional structure of a HypB

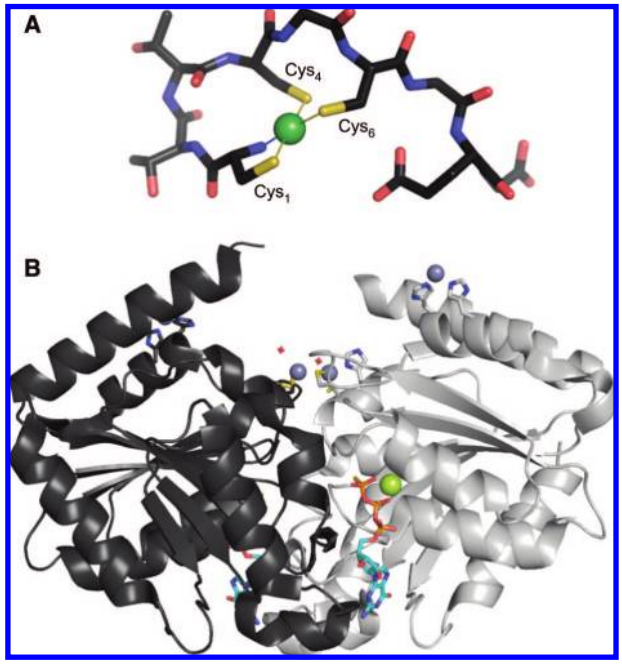

Figure 4. Metal-binding sites of hydrogenase accessory protein HypB. (A) Structure of the high-affinity nickel-binding site of *E. coli* HypB derived from density functional theory (DFT) molecular modeling of the N-terminal 9 residues. The nickel is bound to the terminal amino group and the three cysteines of the CXXCGC motif, as determined by mutagenesis, spectroscopy, and chemical modification experiments. The calculated bond lengths of the energy-minimized structure are in excellent agreement with those measured by XAS. (B) Structure of HypB from *M. jannaschii* (pdb 2HF8). Two zinc ions (silver) are bound at the interface of the protein dimer to conserved Cys95 and Cys127 from one monomer and Cys95, Cys127, and His96 from the other, as well as several solvent molecules. The corresponding *E. coli* numbering is Cys166, His167, and Cys198. This homologue does not have the N-terminal sequence found in the *E. coli* protein. Zinc ions are also bound on the surface to His100 and His104 as well as residues from a molecule of the adjacent crystal contact (not shown). The color scheme is magnesium, lime; zinc, silver; oxygen, red; sulfur, yellow; phosphorus, orange; carbons of the GTPγS, cyan; and nitrogen, blue. The picture was generated by using MacPymol.

homologue with this sequence has not yet been reported, the location of the nickel site at the end of the protein led to speculation that it is involved in nickel transfer.[163,164] Furthermore, the incomplete conservation suggests that this tight nickel-binding activity is only required in the specific cytosolic conditions of some organisms. For example, this sequence would allow HypB to compete for the metal ion and ensure that sufficient amounts are directed to the hydrogenase enzymes if nickel availability is limited. This model requires that the nickel can be released from the HypB site, and there is evidence that other factors involved in nickel insertion may trigger such an event (section 3.1.3).[165]

In addition to the N-terminal nickel-binding sequence, *E. coli* HypB also binds metal at a site composed of highly conserved cysteine and histidine residues embedded in the C-terminal GTPase domain.[160,162] Zinc binds with a K_D of 1 μM, an order of magnitude tighter than nickel,[162] and mutagenesis of the ligands prevents hydrogenase biosynthesis in *E. coli*.[104,164] The crystal structure of the HypB homologue from *Methanocaldococcus jannaschii* (Figure 4B) reveals a dinuclear zinc center bound at the interface of a protein dimer,[160] and XAS of the *E. coli* protein suggests that it hosts a similar complex.[164] One of the metal ligands is a residue in the same loop as the switch II GTPase motif, which contacts the γ-phosphate and connects GTP hydrolysis to a functional protein conformational change in GTPases,[166] suggesting that metal binding and the GTPase activity of HypB are coupled.[160] However, mutagenesis of the metal ligands did not produce a detectable effect on the GTPase activity of the isolated protein,[104,162] so the nature of this link remains to be defined, as does the possible communication with the N-terminal metal site.[164]

3.1.2. HypA

HypA and its homologue HybF bind nickel with micromolar affinity at a site that includes the conserved His2,[159,167,168] and disruption of this activity in vitro also inhibits hydrogenase maturation in vivo.[159,168] A heterodimer between HypA and HypB was detected with both the *E. coli* and *H. pylori* proteins,[159,167] so it is possible that the two factors cooperate for nickel insertion in vivo, although a HypB−HybF complex has not yet been observed,[168] and complex formation was independent of nickel and GTP.[159] Surprisingly, HypB and HypA also modulate nickel delivery to the urease enzyme in *H. pylori* (section 3.2.3),[151] although mutations of either gene do not completely abrogate urease activity like they do for hydrogenase, and this connection was not observed in the cyanobacterium *Synechocystis* sp. PCC 6803.[150]

HypA also binds zinc with nanomolar affinity in a distinct tetrathiolate site that is likely composed of cysteines from two conserved CxxC motifs.[167−170] This type of coordination environment is often found for structural zinc ions in well-defined protein domains that mediate interactions with other biomolecules,[171−173] so it is not surprising that the presence of zinc influences the quaternary structure of the protein.[167] Along the same lines, HypA could serve as a scaffold for the other components of the nickel insertion complex and the hydrogenase large subunit. This model would explain why *E. coli* employs two homologues, HypA and HybF, for the biosynthesis of different hydrogenases,[168] but such a complex has not yet been observed. Although the presence of zinc does not seem to affect nickel binding,[167,168] the reverse is not true because XAS demonstrated that nickel influences the coordination environment around the zinc,[170] suggesting that there is a functional connection between the metal sites.

3.1.3. SlyD

The *E. coli* HypB protein is unlike other HypB homologues because it does not have a histidine-rich sequence (discussed above), so additional factors were sought that could contribute to nickel storage and hydrogenase biosynthesis in this organism.[174] A genomic tagging method[175] was used to pull HypB out of cell extracts in a complex with SlyD,[174] an interaction that was also observed in *H. pylori*.[176] SlyD is a member of the FK506-binding protein (FKBP) family of peptidyl-prolyl isomerases (PPIases),[177,178] and it also has an unusual 50-residue C-terminal domain containing 15 histidines, 6 cysteines, and 7 carboxylate amino acids. This domain can bind multiple metal ions and mediates nickel inhibition of the PPIase activity.[178,179] Deletion of *slyD* in *E. coli* results in a reduced hydrogenase phenotype that is suppressed by excess nickel in the growth medium, indicating that SlyD contributes to the maturation process.[174] In support of a role for SlyD in nickel storage, the protein influences nickel accumulation in *E. coli*,[174] and a truncated

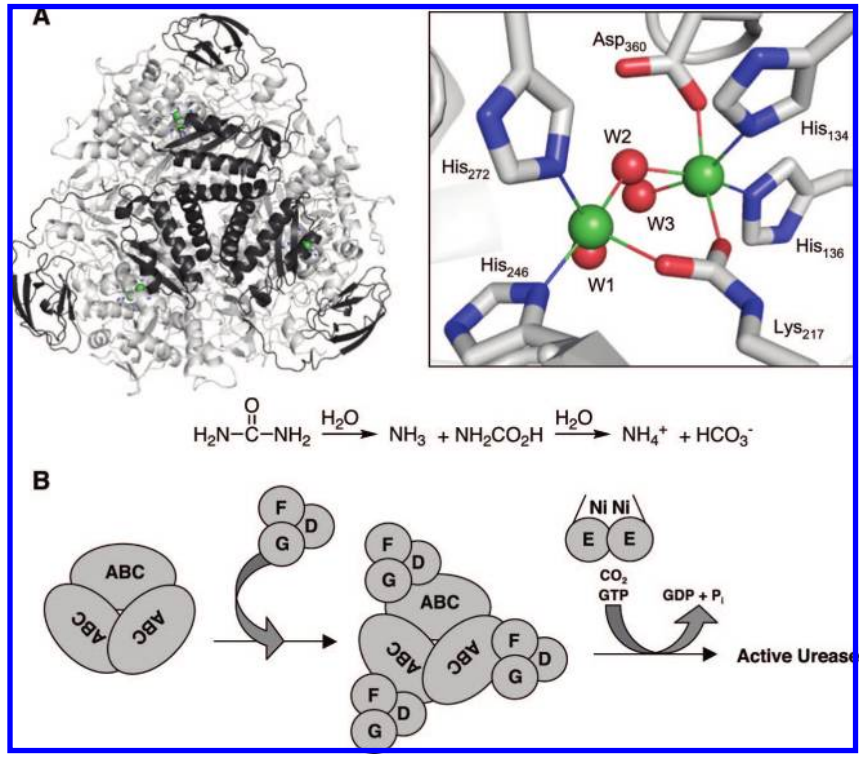

Figure 5. Structure and assembly of urease. (A) Structures of the urease from *K. aerogenes* (left, pdb 1FWJ) and the dinuclear active site (right). The UreC subunits (α), which contain the active sites of the $(UreABC)_3$ structure, are depicted in light gray, and the UreA (γ) and UreB (β) subunits are in dark gray. The two nickel ions are bridged by a carbamylated lysine, and three water molecules are also present. The color scheme is nickel, green; oxygen, red; and nitrogen, blue. The pictures were generated by using MacPymol. (B) Working model for the assembly of the active site on the urease precursor protein. All proteins in the sketch are Ure proteins.

SlyD lacking the metal-binding domain cannot complement the hydrogenase deficiency of the Δ*slyD* strain.[165] However, when SlyD forms a complex with HypB, it dramatically increases the rate of nickel transfer from the N-terminal high-affinity site of HypB to a small-molecule chelator,[165] thus implying a more active role for SlyD in hydrogenase biosynthesis, and this effect requires the metal-binding domain of SlyD. In contrast, mutations that reduce the PPIase activity of SlyD do not result in a correlated decrease in hydrogenase production or metal release from HypB.[180] A recent NMR structure of SlyD* with an abbreviated metal-binding domain (residues 1−165, lacking the C-terminal 31 amino acids) revealed two well-folded domains that are independently oriented, but the part of the metal-binding domain present in the protein was not ordered.[181] Furthermore, titration experiments demonstrated that several residues critical for complex formation with HypB are also involved in binding a variety of peptide and protein substrates.[165,181] SlyD homologues are only found in some prokaryotes and have variable degrees of conservation in the metal-binding domain,[182] so whether SlyD fills a role in hydrogenase biosynthesis that is only required in specific organisms or if it can be replaced by other factors remains to be determined.

3.1.4. Additional Factors

In certain systems, there are also extra accessory proteins that are needed for hydrogenase biosynthesis. For example, several organisms express a protein called HypX (or HoxX) with sequence similarity to the tetrahydrofolate-dependent enzymes.[183−185] In *C. necator*, it is thought to be responsible for the unusual oxygen resistance of the soluble NAD-dependent [NiFe]-hydrogenase produced by this organism.[186] The oxygen-tolerant enzyme has an active site with two additional CN ligands,[186,187] at least one of which is nickel-bound and delivered by HypX. The multipurpose chaperonins GroEL and GroES also contribute to metallocenter assembly of the *E. coli* hydrogenases by directly interacting with the assembly components.[117,188]

3.2. Urease

Urease has a proud place in biochemical history as the first enzyme to be deliberately crystallized,[189] an event that helped to prove in 1926 that isolated "enzyme" catalysts are pure proteins that operate without any additional factors. Ironically, 50 years later the same protein served as the first example of an enzyme that requires nickel for catalysis.[190] The X-ray crystal structure of urease from *Klebsiella aerogenes*,[191] as well as subsequently from several other organisms (reviewed in refs 2 and 192) revealed a dinuclear nickel cluster bound to histidines and an aspartate, along with a bridging carboxylate group donated by a carbamylated lysine (Figure 5). The deeply buried active site also contains several water molecules, at least one of which is required for the catalytic hydrolysis of urea to produce ammonia as well as carbamic acid, which spontaneously decomposes at physiological pH into carbonic acid and a second molecule of ammonia. A variety of other substrates are also hydrolyzed by urease, although none with the efficiency of urea.[193] This enzyme has a critical role in the nitrogen metabolism of many organisms and is a key participant in the global nitrogen cycle.[7,192,194−196] It has agricultural importance because it allows plants to use urea, either recycled or from externally applied fertilizer, as a nitrogen source,[194] and it may also serve in plant defense as an insecticide.[1] In addition, urease is a virulence factor in a variety of pathogenic microorgan-

isms, and it often contributes to the diseased state caused by infection.[195,197−199] For example, the ammonia produced by urease is thought to protect bacteria as they colonize or pass through the acidic environment in the stomach by neutralizing the local environment.[197,198,200]

Four accessory proteins, UreDEFG, are typically involved in the biosynthesis of the urease metallocenter (Figure 5)[2,90,196,201] and are required for full activation of urease when the genes are heterologously expressed in *E. coli*,[61,202−207] although very low levels of activity are detectable in their absence.[208] The only exception found so far is the *Bacillus subtilis* urease that is activated in the absence of any clear accessory protein genes in the genome.[208] The apoprotein lacking carbamylation is completely organized into the same structure as holo-urease,[209] implying that, in addition to delivering nickel and carbamylating the active-site lysine, the maturation pathway must either hold the protein in an open state following translation or it includes an unfolding event to expose the active site pocket. The latter model was recently supported by evidence for a flexible hinge region in UreB that is important for urease activation, possibly allowing a domain shift that opens up access to the active site of the precursor protein.[210,211] This manipulation of the urease structure may also be influenced by the ubiquitous folding chaperone proteins GroEL/GroES that play a role in urease enzyme production.[212,213]

A series of experiments performed by the group of Hausinger with the *K. aerogenes* urease, as well as others, produced a picture of the urease maturation complex.[2,196] In vitro, apourease can be partially activated without the accessory proteins upon addition of nickel and a source of CO_2,[214] but if nickel is added first, then the production of active protein is blocked.[214,215] The level of activation increases in the presence of UreD (called UreH in *H. pylori*),[205,214] which forms a direct complex with the enzyme protein.[216,217] UreF interacts with UreD through its C-terminus,[217−219] and the addition of UreF to the UreD-apourease complex reduces the amount of bicarbonate necessary for activation and prevents inactivation by nickel,[220] suggesting that UreF controls a conformational change around the urease active site, a model supported by several biophysical studies.[210,211] A complex containing UreDFG-apourease can also be isolated,[221,222] and it is proposed to be the functional unit in urease activation.[2] The exact functions of the accessory proteins are not known, but the properties of UreG and UreE implicate these proteins as a source of energy and nickel, respectively, in the urease maturation process.

3.2.1. UreG

Like HypB, UreG is a member of the P-loop family of GTPases,[154] and on its own, NTP hydrolysis by UreG is either very weak or undetectable.[155,223−225] It has been proposed on the basis of modeling studies that UreF serves as a GTPase-activating protein when in a complex with UreG,[226] but this has not yet been demonstrated experimentally. Mutagenesis of a nucleotide-binding motif in UreG prevents the formation of the UreDFG-urease complex and activation of urease in vivo.[223] Furthermore, the addition of GTP, but not a nonhydrolyzable analogue, results in a dramatic enhancement of urease activation by the UreDFG complex and physiologically relevant concentrations of bicarbonate.[222] How GTP binding and hydrolysis contribute to urease biosynthesis are questions that remain to be answered.

UreG also has a low-affinity metal site, and various homologues bind 1−2 zinc ions per dimer with a K_D of 0.3−42 μM[224,227] and nickel with significantly weaker affinity. Predicted zinc residues include a completely conserved CxH sequence that is in the same location with respect to the GTPase motifs as some of the zinc ligands of HypB, but only a 10-fold weaker affinity for zinc was measured by isothermal titration calorimetry (ITC) upon mutagenesis of both residues in the *H. pylori* UreG.[227] Solution studies of UreG from several organisms reveal that portions of the protein are in considerable flux,[224,225,227,228] and it was suggested that UreG is an inherently disordered protein.[229] Binding of a cofactor such as zinc could activate a disorder-to-structured transition and stabilize the protein in a functional conformation.[224,225] In support of this model, zinc induces dimerization and other structural transformations of *H. pylori* UreG.[227]

3.2.2. UreE

Whether or not UreE plays a crucial role in urease maturation depends on the organism.[61,202,203] In several cases, deletion of *ureE* produces a urease-deficient phenotype that is complemented by increasing the amount of nickel in the growth media,[230,231] suggesting that it acts as a nickel metallochaperone that delivers the nickel to the urease activation complex. However, nickel does not complement *ureE* mutations in several *Helicobacter* species.[232,233] A model involving protein-directed nickel transfer from UreE is supported by the observation that UreE is required for full in vitro activation of the *K. aerogenes* urease by the UreDFG complex and that it is competent even in the presence of chelators.[234] Furthermore, the UreDFG−urease complex was recently pulled out of *H. pylori* cells with a small amount of UreE bound.[176] It is interesting to note that, under certain conditions, UreE is actually an impediment to urease biosynthesis. UreE inhibits the low level of urease activation that can occur in vitro in the absence of the other accessory proteins.[235] Furthermore, it also blocks urease activation in the fully reconstituted system if it is in excess of the total nickel.[234] These observations demonstrate that UreE can compete with urease for nickel and does not simply deliver nickel to the isolated enzyme precursor. In addition, the enhancement of urease activation by UreE, which forms a complex with UreG,[218] is only observed in the presence of GTP,[234] further highlighting the cooperative nature of this pathway. A gene for *ureE* could not be found in a few organisms such as *Mycobacterium tuberculosis* and several plant species,[206,236] and in these cases the function of UreE may be replaced by a version of UreG that has an extra cluster of histidines at the N-terminus.[225,237]

K. aerogenes UreE has a histidine-rich C-terminal tail, and it binds 5−6 nickel ions per dimer with an apparent K_D of 10 μM in distorted octahedral sites composed of O/N donor ligands including multiple histidines.[230,238,239] The histidine-rich sequence is not conserved,[240] and only an incremental loss of urease activation was observed upon truncation of this C-terminal region from the *K. aerogenes* protein (H144*).[230] UreE proteins lacking the His-rich tail still bind 1−2 metal ions per dimer with half saturation in the low micromolar range.[230,239,241−244] Other metals can compete with nickel, particularly Cu(II) and Zn(II),[230,238,244] but they have

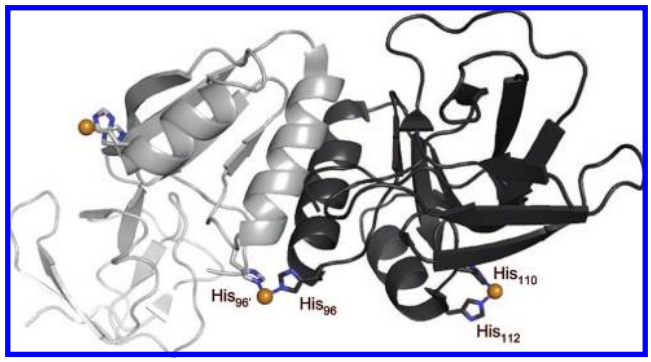

Figure 6. Structure of the UreE urease accessory protein from *K. aerogenes* (pdb 1GMW). Three copper ions (tan) are bound to the two monomers (different shades of gray), with one ion bound to His96 from each subunit at the dimer interface and the other two on opposite surfaces bound to His110 and His112. The protein is a truncation lacking the C-terminal histidine rich tail (H144*). The picture was generated by using MacPymol.

distinct coordination environments,[239] suggesting that nickel may have a selective allosteric effect on the protein and its activity. A mechanism of nickel selectivity is important in vivo because if other transition metals are loaded onto apourease in vitro,[215,245] little if any activity is detected.[245,246]

The strikingly similar crystal structures of the truncated *K. aerogenes* UreE (H144*) (Figure 6) and *Bacillus pasteurii* UreE revealed two distinct domains separated by a flexible linker along with a poorly resolved C-terminus.[240,247,248] The N-terminal domain resembles the peptide-binding domain of heat-shock proteins, suggesting that it is involved in protein−protein interactions with other components of the urease metallocenter assembly complex. Elimination of this domain, however, only results in a partial decrease in the influence of UreE on urease activation.[249] The dimerization domain is structurally related to the Atx1 copper metallochaperone, although the metal sites do not correspond to those of Atx1. Both UreE structures have one metal ion bound at the interface of two (*K. aerogenes*) or four (*B. pasteurii)* monomers, as predicted by solution studies,[242,250] with each monomer contributing the same histidine as a ligand. This histidine (His96 in the *K. aerogenes* protein, His100 in the *B. pasteurii* protein) is completely conserved and it is critical for the role of UreE in urease activation in vivo[250] and in vitro.[234] Given the apparent open coordination sites of the metal bound to the protein dimer, it was suggested that the C-terminal tail, which almost always contains at least one histidine,[240] could close up around the metal in the absence of additional partner proteins.[248] A subsequent solution study provided evidence that histidines in the flexible C-terminus are involved in nickel binding.[251] Furthermore, XAS of *B. pasteurii* UreE loaded with two nickel ions per dimer was best fit to a dinuclear Ni−O−Ni moiety with two histidine ligands on each nickel, one of which must come from the C-terminal region.[243] The authors proposed that this preassembled cluster could be transferred intact to the urease apoprotein, a model that has also been proposed for the *K. aerogenes* system[239] and one that opens up many questions such as how the site is first synthesized on UreE. The crystal structure of the *K. aerogenes* dimer has an additional copper ion bound to the surface of each monomer to nonconserved histidines[247] that are important for nickel binding in vitro[250] and influence the rate of urease activation in vivo.[235] In contrast, mutating His96 in the *K. aerogenes* protein does not appreciably affect the stoichiometry or affinity of nickel binding to recombinant protein.[235,250]

Careful ITC experiments demonstrated that the number and types of metal-binding sites on UreE vary depending on the protein concentration, presumably due to the influence on quaternary structure.[244] This relationship may reflect differences in the metal-binding activity of UreE when it is an isolated dimer versus in the urease activation complex. These experiments, in conjunction with the previous biochemical and structural studies, lead to a scheme in which the nickel ions are bound at the periphery of UreE in the resting state, and the conserved histidine at the dimer interface only gets involved upon nickel delivery to the urease protein.[244] This general scheme does not exclude the possibility that UreE binds and delivers a dinuclear nickel cluster,[243] and further information will be gained if UreE is investigated in a system with all of the urease maturation components. Furthermore, a surprising conclusion was that binding of two nickel ions to the truncated *K. aerogenes* and *B. pasteurii* UreE dimers occurs through different processes that are enthalpically and entropically driven, respectively,[244] suggesting that the chemical mechanism of nickel binding, and thus possibly nickel transfer, is not conserved.

3.2.3. Connection to [NiFe]-Hydrogenase Biosynthesis

In addition to the hydrogenase deficiency, a significant decrease in urease activity was observed in Δ*hypA* or Δ*hypB* strains of *H. pylori* and *H. hepaticus*.[151,155,233] Urease activity was restored in *H. pylori* upon supplementing the growth medium with nickel, and the interruption of genes for other hydrogenase accessory proteins did not affect urease activity,[151,252] suggesting that HypA and HypB boost nickel delivery to the urease enzyme. The inverse relation is not true because mutations in *ureE* or *ureG* do not affect hydrogenase activity.[155,233,241] In contrast, nickel supplementation did not significantly restore hydrogenase or urease activity in the *H. hepaticus* mutants,[233] so there may be subtle differences in how these proteins function or access nickel in the two related organisms.

The connection between the urease and hydrogenase biosynthetic pathways was strengthened by the observation that HypA interacts directly with UreE in a nickel-independent fashion.[232] Complex formation was also observed between *H. pylori* HypB and tagged UreG in cell extracts,[176] along with all of the other urease accessory and structural proteins, and in the reverse experiment, tagged HypB was used to pull out the urease enzyme proteins. In addition, the urease enzyme subunit UreA was found in association with SlyD,[176] which has a role in *E. coli* hydrogenase production (section 3.1.3).[174] The possibility that SlyD contributes to urease biosynthesis was supported by the observation that a *hypAslyD* double mutant of *H. pylori* exhibits significantly less urease activity than either of the single-component mutants,[253] suggesting that SlyD plays a role overlapping that of HypA.

HypA/HypB involvement in *H. pylori* urease biosynthesis may reflect a strained nickel delivery system, especially considering that urease production can reach up to 10% of the total protein in this organism[254] and *H. pylori* UreE only binds one nickel ion, unlike many of its homologues.[201] In agreement with this hypothesis, adding unnatural His-rich sequences to the C-terminus of *H. pylori* UreE augmented the nickel capacity of the protein in vitro and urease activation in a Δ*hypA* or Δ*hypB* strain.[241] However, no complementation of the hydrogenase deficiency was ob-

served, indicating that the nickel sequestered by UreE is targeted to urease.

3.2.4. Additional Factors

Neither UreE nor HypB of *H. pylori* have the His-rich sequences found in other homologues, and the C-terminal domain of *H. pylori* SlyD has a stretch of metal-binding residues that is only about a third of the length of that in the *E. coli* protein. Given the high demand of *H. pylori* for nickel, it is perhaps not surprising that the organism expresses several other proteins that bind to this metal and contribute to nickel homeostasis.[2,201] One example of such a protein is the GroES heat-shock protein HspA, which has an unusual C-terminal sequence containing eight histidines and four cysteines.[213] HspA and the GroEL homologue HspB, which may be antigens for the immune response to *H. pylori* infections,[213] improve the urease activity measured upon heterologous expression in *E. coli*.[212,213] HspA is a heptamer that binds two nickel ions per monomer with an apparent K_D of $1-2\ \mu$M as well as additional ions with substantially weaker affinity.[212,255] The observation that HspA provides nickel resistance to *E. coli* is consistent with a role in nickel storage,[255] but as mentioned above it is feasible that the chaperones also have a role in controlling the conformation of urease during metallocenter assembly in vivo. Defined disulfide bridges were characterized in HspA,[256] which is localized to both the cytoplasm and extracellular milieu,[257] but how these structures affect nickel binding is not known.

Two other *H. pylori* proteins that bind nickel are called Hpn and Hpn-like. The 60-residue sequence of Hpn is striking because it includes 28 His, 9 Glu/Asp, and 4 Cys.[258] Hpn-like is slightly bigger and is just under one-third histidine residues and almost one-half glutamines. Both proteins form large aggregates and bind multiple nickel ions per monomer with apparent affinities in the low micromolar range,[259,260] and both provide resistance to nickel toxicity.[259−262] Experiments with bacteria grown under low nickel conditions revealed a significant increase in urease activity upon deletion of *hpn* or *hpn-like* but no effect on hydrogenase,[262] suggesting that these proteins compete for nickel with the urease accessory proteins, perhaps for the purpose of long-term storage.[201] This hypothesis is consistent with the observation that adding a nickel chelator to the medium brought the urease activity in the mutants back down to wild-type levels.[262]

HspA and Hpn also bind bismuth,[255,263,264] and while Hpn provides resistance to bismuth compounds,[261,264] HspA increases the bismuth sensitivity of *E. coli*.[255] As such, HspA is proposed to contribute to the effectiveness of bismuth-containing drugs in treating *H. pylori* infections. In contrast, the presence of Hpn did not affect the sensitivity of *H. pylori* to Co(II), Zn(II), or Cu(II),[261,262] although the protein binds zinc with a similar affinity as nickel.[264]

3.3. Superoxide Dismutase

Superoxide dismutase (SOD) enzymes catalyze the disproportionation of the cytotoxic superoxide radical into hydrogen peroxide and oxygen gas by cycling through reduction and oxidation reactions, thus protecting the cell from oxidative damage.[265] There are several distinct classes of SOD that utilize different metals, and NiSODs have been found in the genus *Streptomyces*, some marine cyanobacteria, and a few other organisms.[266−271] The crystal structures of

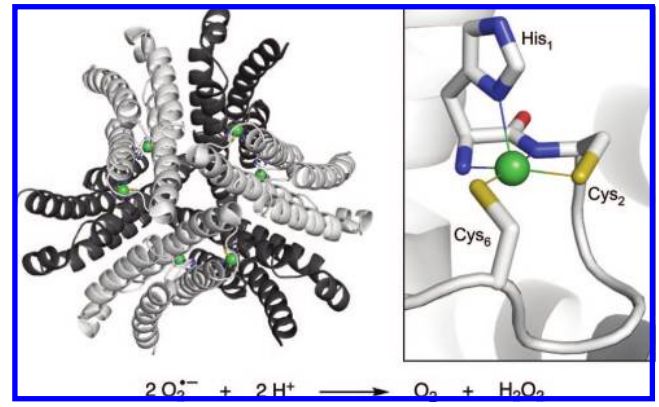

Figure 7. Structure of the nickel superoxide dismutase from *Streptomyces coelicolor* (left, pdb 1T6U) and the nickel-containing active site (right). Each monomer of the hexameric protein has a nickel ion bound to the N-terminal His-Cys-X-X-Pro-Cys sequence. The side chain of the histidine is an axial ligand for Ni(III) (shown), and in the reduced state it swings around, leaving a square-planar Ni(II) complex (not shown). The color scheme is nickel, green; oxygen, red; sulfur, yellow; and nitrogen, blue. The pictures were generated by using MacPymol.

NiSOD (Figure 7) reveal that Ni(III) is bound in a square-pyramidal site to an N-terminal His-Cys-X-X-Pro-Cys-Gly-X-Tyr motif, with the two cysteines, the amino-terminal nitrogen, and a backbone nitrogen (position 2) serving as planar ligands and the histidine imidazole serving as an axial ligand.[272,273] The reduced form has Ni(II) in a square-planar site due to loss of the imidazole ligand as predicted by spectroscopy,[274−276] although this change may not occur during catalysis.[277] The cytosolic NiSOD protein is expressed with a leader sequence that must be cleaved in order to expose the histidine as the first residue in the processed protein.[268,276,278] In the absence of nickel, the N-terminal region is disordered,[272] and it was suggested that proteolytic cleavage is coupled to nickel binding, similar to the nickel-dependent processing observed during the biosynthesis of the [NiFe]-hydrogenases. A putative peptidase with homology to serine proteases has been annotated downstream of *sodN* genes[266,270] and is required for heterologous expression of active enzyme.[266] Other accessory proteins may include a PPIase to isomerize the amide of Pro5.[266,270,272] In an effort to find a metallochaperone that delivers nickel to NiSOD, extracts of *Streptomyces seoulensis* were fractionated over a nickel-NTA column.[279] The protein that was isolated, CbiXhp, has a histidine-rich tail, but it is homologous to cobalt-chelatases and the gene is part of a cobalamin biosynthetic operon, so its potential role in NiSOD production is not yet clear.

3.4. Carbon Monoxide Dehydrogenase/Acetyl-CoA Synthase

Carbon monoxide dehydrogenase (CODH) catalyzes the redox reaction that interconverts carbon monoxide and carbon dioxide, and allows organisms to use CO as a carbon source.[2,4,280,281] The crystal structures of several CODHs reveal a protein that contains multiple metal clusters (Figure 8),[282−287] including three $[Fe_4S_4]$ clusters that shuttle electrons to or from the nickel-containing C-cluster at the active site. Although there are small differences between the structures in terms of the ligands and metal geometries of the C-cluster, it is essentially an unusual Fe_3S_4 center bridged to a fourth iron and a nickel ion (reviewed in refs 281, 288, and 289).

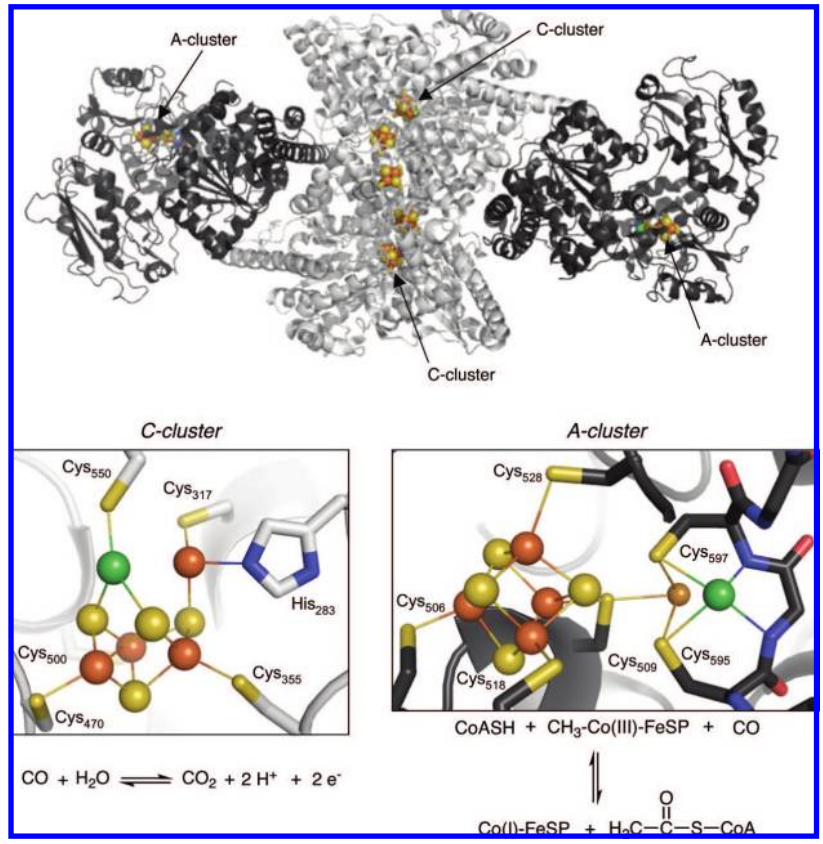

Figure 8. Structure of the carbon monoxide dehydrogenase/acetyl-CoA synthase from *Moorella thermoacetica* (top, pdb 2Z8Y). The two CODH subunits (light gray) contain a total of five metal clusters including two C-clusters. The nickel ion in the C-cluster (bottom, left) also has a small molecule ligand (not shown), and the bridging of ligands between the nickel and the unique iron center varies between the homologues that have been structurally characterized. Two ACS subunits (dark gray), containing one A-cluster each, flank the CODH dimer. The proximal metal in the A-cluster of the structure shown in the bottom right is copper (tan), but the functional metal is probably nickel, and electron density suggests that there is an additional small molecule coordinated to this metal. The methyl group for the biosynthesis of acetyl-CoA at the A cluster is supplied by the autonomous corrinoid−iron−sulfur protein (CoFeSP). The color scheme is nickel, green; iron, orange; copper, tan; sulfur, yellow; oxygen, red; and nitrogen, blue. The pictures were generated by using MacPymol.

As for hydrogenase and urease, the biosynthesis of CODH in *Rhodospirillum rubrum* involves several accessory proteins that are required in the absence of supplemental nickel, including an NTPase (CooC) and a putative nickel metallochaperone (CooJ).[290,291] CODH purified from nickel-deficient cells has intact iron centers and can be reconstituted in vitro by the anaerobic addition of $NiCl_2$,[292] a process inhibited by other divalent metal ions,[293] suggesting that the metallocenter is assembled directly on the enzyme protein in distinct steps. Kinetic analysis indicates that nickel activation occurs in two stages, nickel binding followed by a slight reorganization.[294] The weak K_D estimated for nickel binding in vitro, 0.7−0.8 mM,[293,294] suggests that this process is facilitated by accessory proteins in vivo. Like HypB and UreG, CooC has a nucleotide-binding P-loop motif, although ATP is a slightly better substrate than GTP.[290,295] Upon generation of a CooC mutant with decreased ATPase activity in vitro, nickel insertion and activation was blocked in vivo.[295] CooJ has a histidine-rich sequence at the C-terminus, but as in other systems, this region is dispensable;[290,295] it binds four nickel ions with an apparent K_D of 4 μM as well as other divalent metal ions.[296] A third gene was identified in between *cooC* and *cooJ* and a weak homology between the 66-residue CooT and HypC was noted, but its role remains unclear.[290,291,295] Furthermore, unlike CooC, homologues to CooJ and CooT are not found in many CODH-expressing organisms, so they may fulfill specialized functions.[297]

Some CODHs are components of a larger enzyme complex where they are coupled to the biosynthesis of acetyl-CoA (acetyl-CoA synthase, ACS) or decarbonylation of acetyl-CoA (acetyl-CoA decarbonylase, ACDS).[280,298] This chemistry occurs at a metallocenter called the A-cluster (Figure 8), which is separated from the C-cluster by a long, enclosed channel through which CO can travel.[281,289,299,300] The A-cluster is an $[Fe_4S_4]$ cluster linked to a binuclear metal site by a bridging cysteine.[282,284,301] The metal distal to the iron−sulfur cluster is a nickel ion bound in a square-planar geometry to two backbone nitrogens and two cysteine side chains that also bridge to the proximal metal. This proximal metal site is occupied by copper, zinc, or nickel in the various crystal structures,[282,284,301] but biochemical and spectroscopic studies suggest that the physiological enzyme is the Ni−Ni form.[281] Furthermore, loading nickel, but not other metals, into the proximal site influences the quaternary structure of the A-cluster subunit in a manner that correlates with activity, indicating a metal-selective conformational change.[302] A gene for an NTPase called AcsF is found in many organisms containing ACS/CODH,[303] sometimes in addition to a *cooC* gene, and the protein from *Moorella thermoacetica* (previously named *Clostridium thermoaceticum*) exhibits weak ATP hydrolysis.[304] However, this protein is not required for the biosynthesis of the C-cluster or possibly the A-cluster upon heterologous expression in *E. coli*.[304,305]

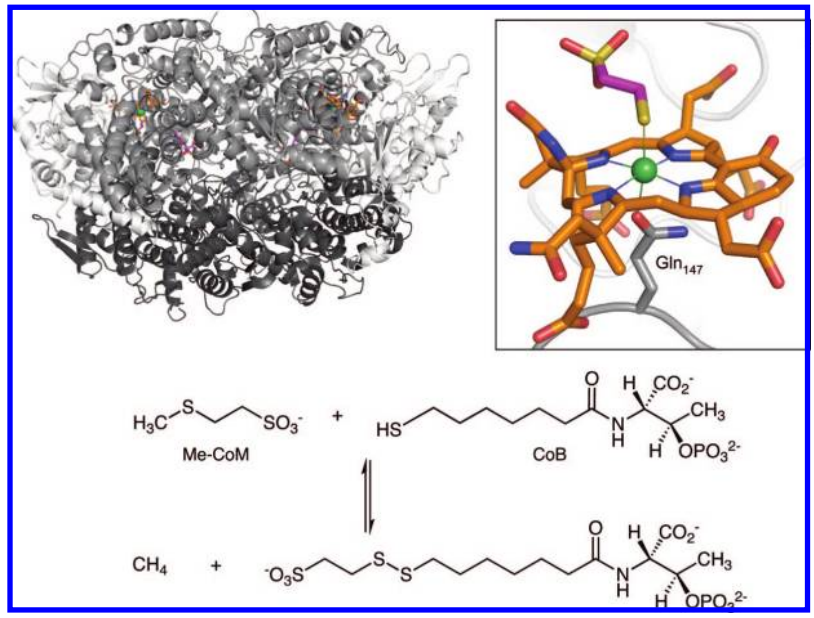

Figure 9. Structures of the methyl-coenzyme M reductase from *Methanobacterium thermoautotrophicum* (left, pdb 1MRO) and the tetrapyrrole cofactor (right). The nickel-containing F430 (orange) is embedded between the α, α′, β, and γ, and α, α′, β′, and γ′ subunits of the hexamer. The axial nickel ligands are Gln147 and coenzyme M. The color scheme is nickel, green; oxygen, red; sulfur, yellow; and nitrogen, blue. The pictures were generated by using MacPymol.

3.5. Methyl-CoM Reductase

Methyl-CoM reductase is the terminal enzyme of methane biosynthesis in methanogenic archaea and has also recently been identified in methanotrophic archaea.[306,307] This enzyme, which makes a significant contribution to the global carbon cycle,[8,9] catalyzes the reduction of methyl-coenzyme M (methyl-2-thioethanesulfonate) with coenzyme B (*N*-7-mercaptoheptanoylthreonine phosphate) as the electron donor to produce methane and a disulfide-linked CoM-CoB. The coenzyme Factor 430 is a nickel-containing corphin (Figure 9) that has an absorbance maximum at 430 nm in its nonphysiological oxidized state and is deeply buried in the multiprotein complex.[8,308,309] The biosynthesis of this prosthetic group proceeds through 5-aminolevulinic acid and urophorphyrinogen III,[308] in the same fashion as with other cyclic tetrapyrroles such as heme and vitamin B12,[310] followed by several modification steps.[2] How the nickel is inserted into the ring structure and how the coenzyme is subsequently embedded into the final form of the enzyme is not clear. In the cases of other metal-containing tetrapyrroles, there are specific chelatases that insert the appropriate metal ion, such as the ferrochelatase involved in heme biosynthesis,[310] so it is likely that there is an accessory protein that performs the analogous function with nickel in this pathway. Nickel-containing hydroporphyrins (acyl tunichlorins) were also isolated from the tunicate *Trididemnum solidum*,[311] and it was suggested that the marine invertebrate may use a chlorophyll degradation product from the alga symbiotic partner as a precursor of the nickel chelate, but very little is known about the biosynthesis or function of this class of nickel biomolecules.

3.6. Glyoxylase I

Glyoxalase I (GlxI) is a cytosolic enzyme that contributes to the detoxification of methylglyoxal and other α-ketoaldehydes.[312] It was thought that all GlxI were zinc enzymes until the discovery that *E. coli* GlxI is inactive when loaded with Zn(II) and is instead most active when bound to Ni(II).[313,314] XAS and X-ray crystallography reveal that the nickel in the resting enzyme is coordinated in an octahedral site composed of two histidines, two glutamates, and two water molecules (Figure 10),[315,316] whereas the inactive zinc-loaded site is five-coordinate. Given that this enzyme is also activated by other divalent metal ions such as Co(II), it is sometimes referred to as the non-Zn(II) GlxI, and it is now clear that this type of GlxI represents a class of enzymes found in multiple prokaryotes.[317,318] No connection between GlxI and any of the known nickel homeostasis factors has yet been found, so if it is a nickel enzyme, then it will be interesting to learn about the mechanism of nickel delivery, especially given that zinc binds more tightly than nickel.[314]

3.7. Acireductone Dioxygenase

Nickel is a cofactor of acireductone dioxygenase from *Klebsiella ATCC 8724* (*K. pneumoniae*),[319] an enzyme in the ubiquitous methionine salvage pathway and the only example of a nickel-containing oxygenase.[2] Surprisingly, iron also binds to the enzyme with the same ligand set of three histidines and a glutamate (Figure 11),[320−323] but it results in a different protein conformation and distinct enzymatic products.[319,320,324] This system raises interesting questions about the delivery pathways of each of the two metals to the precursor enzyme protein and how this process is regulated, but the biosynthesis of this enzyme has not yet been described.

3.8. Additional Nickel Enzymes?

In addition to the eight enzymes described above, there are reports of other proteins that are active with nickel including the monocupin dioxygenase quercetinase from *Streptomyces* sp. FLA,[325] the glycerol-1-phosphate dehydrogenase AraM from *B. subtilis*,[326] and a methylenediurease from a *Burkholderia* species,[2] and it is likely that this list will continue to grow. However, there are examples of proteins that were originally classified as nickel-dependent

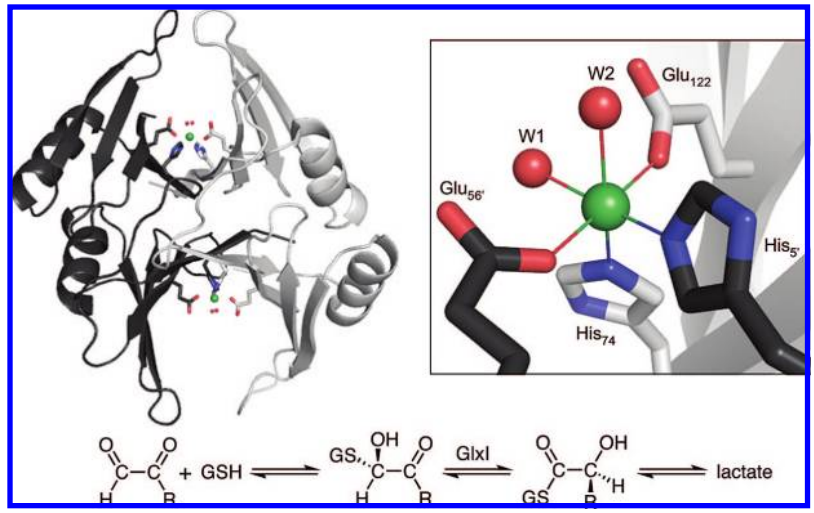

Figure 10. Structure of the glyoxalase I from *E. coli* with two solvent molecules bound (left, pdb 1F9Z) and the enzyme active site (right). The two monomers of the dimeric protein are shown in different shades of gray. The color scheme is nickel, green; oxygen, red; and nitrogen, blue. The pictures were generated by using MacPymol. The reaction between methylglyoxal and glutathione (GSH) is nonenzymatic, and the final step to produce lactate is catalyzed by glyoxalase II.

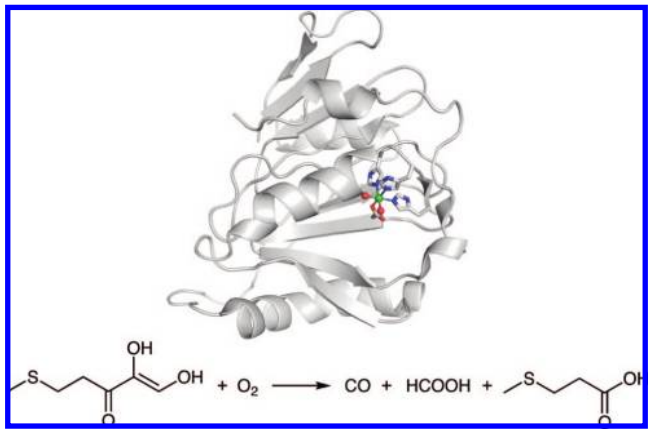

Figure 11. Solution structure of the acireductone dioxygenase from *Klebsiella oxytoca* (pdb 1ZRR). The nickel ion is bound to His96, His98, His140, Glu102, and two water molecules. The color scheme is nickel, green; oxygen, red; and nitrogen, blue. The picture was generated by using MacPymol.

enzymes but were subsequently shown to use a different metal ion in vivo,[2] so some caution is necessary in making a final designation. Furthermore, even in organisms that do not have a known functional requirement for nickel, there are proteins that bind and respond to the metal. For example, although there is circumstantial evidence of a nutritional requirement for nickel in animals,[327−329] no specific function for nickel has yet been identified. However, nickel binds to a variety of mammalian proteins, including the amino-terminal Cu(II)- and Ni(II)-binding (ATCUN) motif found in serum albumins and other proteins,[330] as well as histones,[331] histatins,[332] and perhaps other biomolecules.[333]

4. Regulation

Nickel homeostasis in prokaryotes is strictly maintained by transcription factors that manage the expression of genes encoding proteins responsible for nickel uptake, sequestration, utilization, efflux and detoxification. Many of the systems discussed above are regulated by various cellular metabolites related to their function, but this review will focus solely on nickel-dependent regulators. Upon binding nickel ions, these proteins activate, derepress, or repress the transcription of their target genes (Figure 12). Over the past decade, several nickel-responsive metalloregulators have been discovered and characterized, including NikR, RcnR, NmtR, KmtR, SrnRQ, and Nur (Table 1), and in this section we will review our current knowledge about these cellular components. Furthermore, although metal-dependent regulation at post-transcriptional levels may be important for nickel homeostasis, they are not yet well-defined and will not be discussed in this report.

4.1. NikR

NikR was first described as a nickel-dependent repressor of the *E. coli nikABCDE* genes that encode a nickel-import system (section 2.1.1).[24,334] Sequence alignments revealed many NikR homologues from a variety of archaea and eubacteria species,[335,336] and the biochemical properties of NikR from *E. coli* and *H. pylori* (EcNikR and HpNikR, respectively) have been studied extensively.[337,338] Although they display features adapted to the specific requirements of their host organisms, there are some common properties.

NikR is the only known metal-regulated member of the ribbon-helix-helix (β-α-α, RHH) family of prokaryotic DNA-binding proteins.[335,337,339] Members of this family have an RHH DNA-binding motif in which the N-terminal sequences of two monomers intertwine to produce an antiparallel β-strand that binds to the major groove of specific DNA-recognition sequences.[339,340] NikR also has a C-terminal metal-binding domain. X-ray crystal structures of apo- and nickel-bound EcNikR, HpNikR, and NikR from *Pyrococcus horikoshii* (PhNikR) reveal a tetrameric quaternary structure with two DNA-binding domains on opposite sides of the protein, with each connected by a flexible linker to the central core composed of four metal-binding domains (MBDs) (Figures 13 and 14).[336,341−343] In the crystal structure of holo-EcNikR, one tetramer has four nickel ions bound to conserved residues at the interface between the MBDs of opposing DNA-binding dimers (Figure 13).[341,342] The ligands, consisting of two histidines and one cysteine from one subunit and a third histidine from another subunit, form a square-planar geometry around the metal center (Figure 13), the same type of coordination geometry observed in XAS experiments.[344,345] The crystal structures of PhNikR and

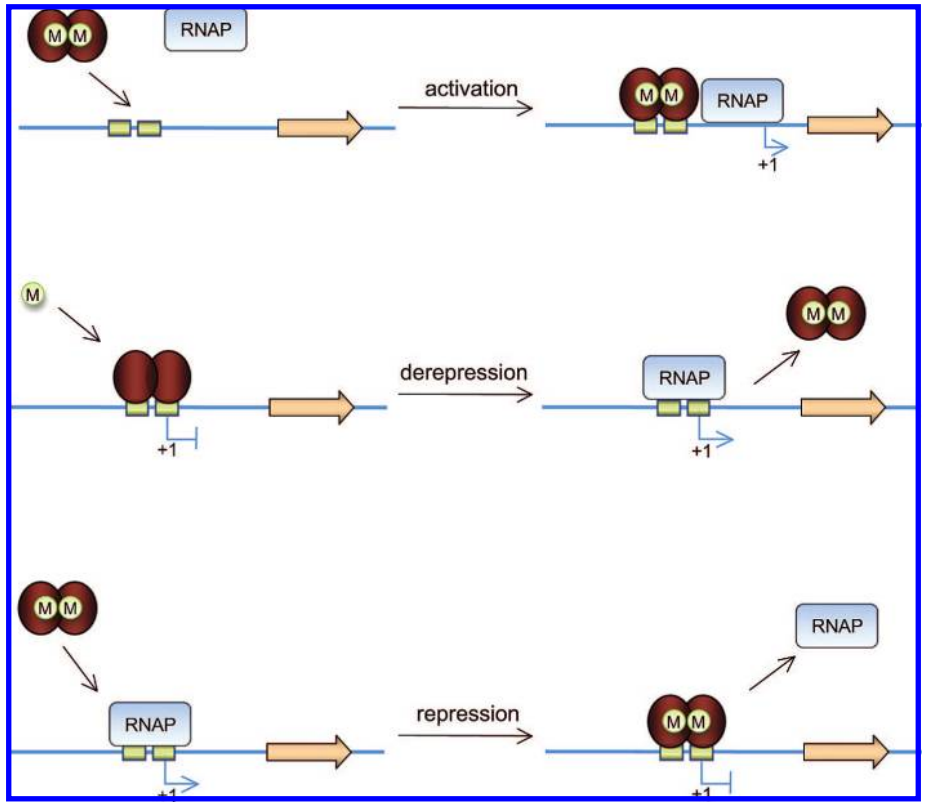

Figure 12. Schematic of potential regulatory responses to nickel. The RNA polymerase (RNAP) binds just upstream of the transcription start site (+1) and initiates transcription. The metal (M) binds to the metalloregulator (ovals), which may not be a dimer as shown here, and causes a conformational change in the protein that influences the interaction with the DNA recognition sequence (yellow boxes) and consequently affects transcription.

Table 1. Summary of Ni(II)-Responsive Transcriptional Regulators

family	regulator	active biological species	functional response to nickel	DNA-binding motif	Ni(II) affinity (M)	coordination geometry	regulated genes
NikR	EcNikR	tetramer	repressor	ribbon-helix-helix	[a]high-affinity site: 9×10^{-13}[346,347] low-affinity site: 3×10^{-8}[351]	square planar octahedral	nickel uptake: *nik*ABCDE
	HpNikR	tetramer	activator and repressor	ribbon-helix-helix	[b]3×10^{-12}[348]	square planar	nickel uptake, metabolism, and storage, iron uptake and storage, stress response, motility genes
RcnR/CsoR	RcnR	tetramer	derepressor	antiparallel four-helix bundle	$<3 \times 10^{-8}$[388]	octahedral	nickel efflux: *rcnA*
ArsR/SmtB	NmtR	dimer	derepressor	winged helix-turn-helix	$<5 \times 10^{-8}$[395]	octahedral	Ni(II)/Co(II) efflux: *nmtA*
	KmtR	dimer	derepressor	winged helix-turn-helix	tighter than NmtR[391]	octahedral	*kmtR* and metal efflux: *cdf*
(ArsR/SmtB)	SrnRQ	4:4	repressor	winged helix-turn-helix	6×10^{-7}[400]		oxidative stress: *sodF*
Fur	Nur	dimer	repressor	winged helix		square planar/ octahedral	oxidative stress: *sodF*, nickel uptake: *nikA*

[a] This binding affinity was measured by using electronic absorption spectroscopy and a competitor. A high nanomolar affinity was measured by using alternative methods.[333] [b] This binding affinity was measured by using electronic absorption spectroscopy and a competitor. Nanomolar affinities were measured by using ITC.[334]

HpNikR also exhibit additional nickel-binding sites and will be discussed below.[336,343] The stoichiometric nickel ions bind to NikR with a dissociation constant estimated to be $\sim 10^{-12}$ M by using EGTA competition assays,[336,346−348] and this picomolar nickel site is referred to as the "high-affinity site". It is possible that the strength of this nickel site has been overestimated, because a weaker nanomolar affinity has been measured by using alternative methods such as ITC.[349,350] However, as described below, holo-NikR binds DNA with a K_D in the low nanomolar range, setting an upper limit for the nickel-binding affinity.

4.1.1. EcNikR

In the presence of stoichiometric nickel (4 nickel ions per tetramer), EcNikR binds to the palindromic operator sequence (GTATGA-N_{16}-TCATAC) in the *nikABCDE* promoter with a K_D of $\sim$5 nM.[346,351,352] This recognition

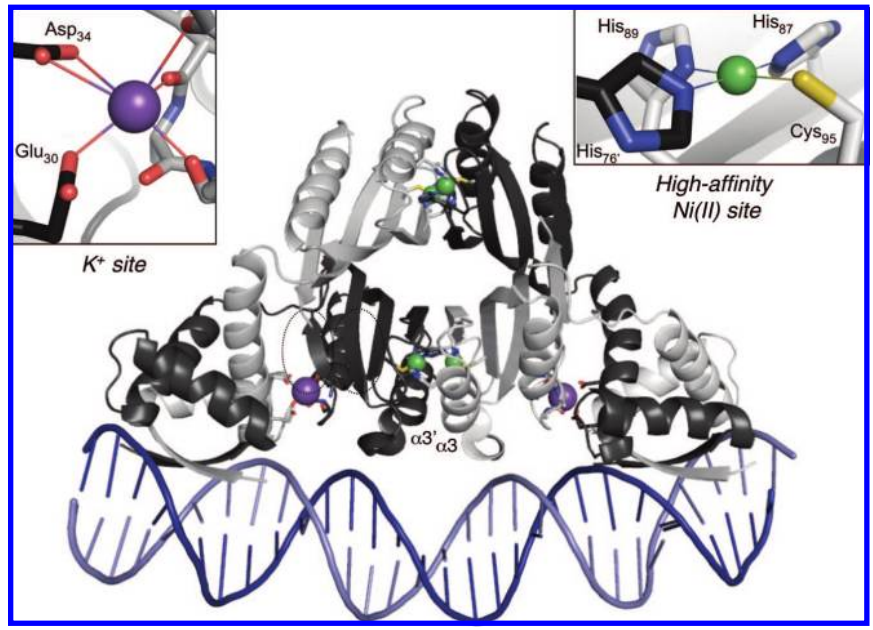

Figure 13. Crystal structure of the *E. coli* nickel-responsive repressor EcNikR in a complex with nickel and DNA. The protein (pdb 2HZV) is shown with each monomer in the tetramer drawn in a different shade of gray. Two ribbon-helix-helix DNA-binding domains flank the central core of four metal-binding domains. The high-affinity nickel ions (green spheres) are coordinated in a square-planar site by His87, His89, and Cys95 of one monomer, and His76′ of the opposing monomer (shown in inset, site rotated for clarity). The other metals (purple spheres) in the DNA complex are best modeled as potassium ions bound to Glu30 and Asp34 of the DBD and three backbone amide oxygens from a loop in the MBD. The α3 loop/helices of two of the MBDs contact the DNA, and these regions are not well-ordered in the apo-structure. The color scheme is nickel, green; potassium, purple; oxygen, red; sulfur, yellow; and nitrogen, blue. The pictures were generated by using MacPymol.

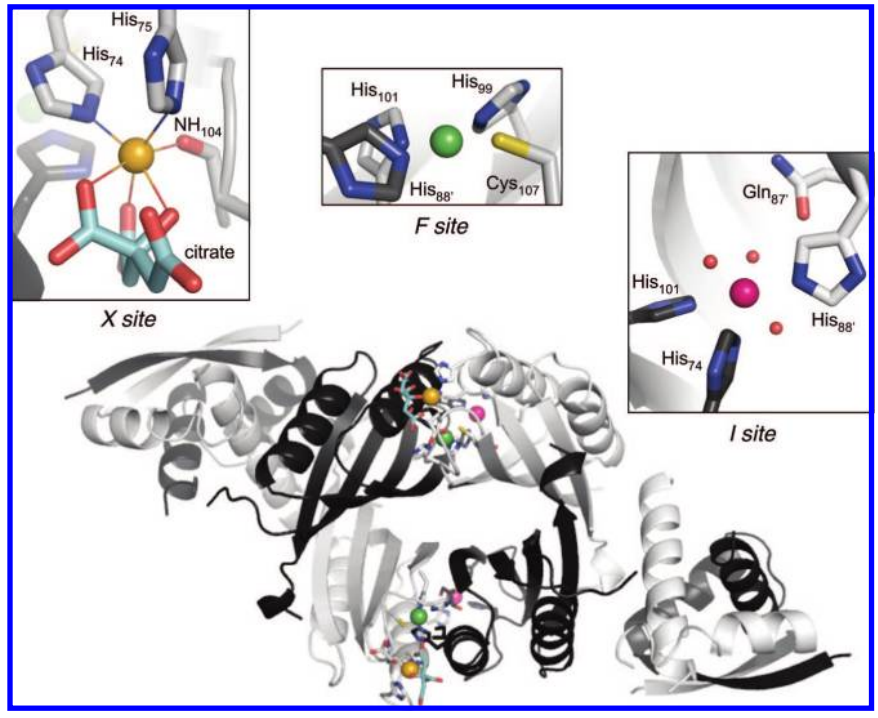

Figure 14. Crystal structure of the *H. pylori* nickel-responsive repressor HpNikR. The holo-protein (pdb 2CAD) is shown with each monomer in the tetramer drawn in a different shade of gray. The two DNA-binding domains are in a trans conformation. Three different nickel sites are observed (insets). The F site (green) is the conserved square-planar site observed in other NikR homologues. The X site (orange) is close to the F site and has a citrate bound from the crystallization solution (light blue). The I sites (pink) are on the opposite side of the protein and are composed of a mixture of residues from the other two sites. The color scheme is nickel, as described above; oxygen, red; sulfur, yellow; and nitrogen, blue. The pictures were generated by using MacPymol.

sequence overlaps the transcription start site,[335,352] so holo-EcNikR likely competes with RNA polymerase and in this manner represses transcription of the *nik* operon. Mutation of the high-affinity site nickel ligands H87A, H89A, or C95A prevents nickel binding, eliminates nickel-induced DNA binding in vitro,[344] and disrupts EcNikR function in vivo.[66] Other metals also bind to EcNikR and induce DNA binding to the *nik* operon, although with at least a slightly weaker DNA affinity than the nickel complex.[347,351] The metal-binding affinities follow the Irving-Williams series (Mn^{2+} <

$Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}$), which is the general trend observed for metal ion affinities of small-molecule ligands,[353] indicating that the high-affinity site is not preorganized for nickel.[347] The hypothesis that the metal-binding site of EcNikR can adapt to the coordination preferences of the individual metal ions was confirmed by both XAS and structural analysis of the protein loaded with several different divalent metal ions.[345,354]

The existence of a second, weaker nickel-binding site in EcNikR is suggested by the observation that the DNA-binding affinity of holo-EcNikR dramatically increases from ~5 nM to ~20 pM in the presence of excess nickel in electrophoresis mobility shift assays (EMSAs) and DNase I footprinting assays.[346,351,352] By titrating holo-EcNikR with nickel ions, a nickel affinity of 30 nM for this second site was indirectly measured.[351] Given an intracellular NikR concentration of 200 nM[346] and that a few "free" nickel ions inside a bacterial cell are in the ~nM range,[355] this low-affinity site is likely to have a physiological function. This model is supported by in vivo reporter assays that revealed that EcNikR mediates two levels of repression on the *nik* promoter, a basal level under low nickel concentrations followed by complete repression at much higher amounts of added nickel.[66] Furthermore, excess nickel induces a much tighter protein−DNA complex than other metals, suggesting that it is a nickel-selective response.[351] XAS of a DNA complex of EcNikR loaded with Cu(II) in the high-affinity site and Ni(II) in the second site revealed a nickel coordinated to six N/O-donors including two imidazoles,[345] but the location of the low-affinity nickel-binding site and the mechanism by which it modulates DNA binding remain unclear.

The X-ray crystal structure of holo-EcNikR bound to the *nik* recognition sequence demonstrated that EcNikR interacts with the DNA through a combination of specific and nonspecific contacts from the RHH DNA-binding domain (DBDs) and nonspecific interactions from the MBDs.[342] This structure also revealed an octahedral potassium-binding site bridging the RHH domain and the MBD,[342] composed of two strictly conserved amino acid side chains, Glu-30 and Asp-34, from the DBD and three backbone carbonyl oxygens of Ile-116, Gln-118, and Val-121, from a loop of the MBD (Figure 13).[342] Mutagenesis studies of E30 and/or D34 indicate that both amino acids are critical for DNA binding by EcNikR.[336,342] The same site was occupied by a Ni^{2+} ion in the structure of PhNikR crystallized with phosphate, which mimics aspects of the DNA.[336] This observation raises the question of whether this site is for K^+ or if it marks the location of the second nickel site predicted by the biochemical studies. Nickel does not replace potassium at this site upon soaking or crystallizing this protein−DNA complex with excess Ni^{2+}.[342] Furthermore, the chemical features of this site, six oxygen ligands with long bond distances (2.7−3.2 Å), are more suited to K^+ than to Ni^{2+},[342,356] and it does not contain the histidine ligands that were observed in the XAS analysis of the second nickel site.[345] Therefore, it is feasible that the potassium site is another structural component of EcNikR's activity, a hypothesis supported by the observation that potassium is critical for the formation of the Ni-EcNikR-DNA complex (S.C.W., D.B.Z., manuscript in preparation).

A mechanism of nickel-induced DNA binding can be proposed based on the biochemical, spectroscopic, and structural studies.[338] In both apo-EcNikR and holo-EcNikR, the DBDs are too far apart to bind the two half-sites of the DNA recognition sequence concurrently.[357,358] However, one difference between the two structures is that, upon binding of stoichiometric nickel, the α3 helix/loop in the MBD makes a disordered-to-ordered transition, allowing nonspecific interactions between the α3 helix/loop and the DNA phosphate backbone. This secondary-structure stabilization by nickel is supported by the crystal structures of both EcNikR and PhNikR,[336,341,342] as well as biochemical studies of EcNikR.[346,347,359] The DBDs can then rotate around to bind to the two half-sites through a large-scale transition stabilized by the DNA interactions.[360] This conformation may put the quaternary structure of EcNikR under strain, but it is bolstered by the nickel ions at the tetrameric interface, partial unwinding of a helix at the linker between the MBD and DBD, binding of potassium between the two domains, and a potential network of contacts linking them.[361] It has been suggested that this DNA complex does not completely block transcription and that it is only when the putative low-affinity site is also filled with nickel that the protein−DNA complex is tight enough for absolute repression; however, this model has not been experimentally proven.

The NikABCDE transporter regulated by EcNikR supplies nickel for the [NiFe]-hydrogenase enzymes,[24,28] and the multilayered regulation of *nikABCDE* is governed by at least two other transcription factors. A decrease in oxygen tension activates FNR (fumarate-nitrate regulator) which upregulates *nikABCDE*,[24,28] while in the presence of excess nickel, EcNikR dominates over FNR and represses *nikABCDE* transcription.[24,66] Nitrate also contributes to *nik* repression through the NarLX two-component system independent of EcNikR,[66] providing a link between hydrogenase enzymes and other mechanisms of anaerobic respiration. Furthermore, metabolites that increase hydrogenase expression cause an increase in the amount of nickel needed to inhibit *nik* transcription, suggesting the presence of a direct link between hydrogenase levels and EcNikR activity.[66] Hence, the nickel-dependent regulation of nickel uptake in *E. coli* is finely tuned to the metabolic needs of the cell. Surprisingly, EcNikR activity does not depend on nickel transported by NikABCDE, suggesting that EcNikR senses nickel imported from another pathway and providing support for separated pools of nickel in the cell.[66]

4.1.2. HpNikR

H. pylori is a gram-negative pathogen that colonizes the gastric mucosa of humans.[97,98] Nickel is of particular importance for *H. pylori* because it expresses two nickel enzymes that are essential for efficient colonization. As mentioned above, one is urease, which produces ammonia to neutralize the acidic microenvironment and is also thought to be important for nitrogen metabolism.[97,198] The other nickel enzyme is a hydrogenase, which allows *H. pylori* to use hydrogen gas produced by normal colonic flora as an energy source.[96,101,362] HpNikR shares 30% identity and 68% similarity with EcNikR[363] and is a key protein in maintaining nickel homeostasis in this organism.[71,201] Unlike EcNikR, which regulates only the *nik* operon, HpNikR is a global regulator that controls the expression of a battery of genes in *H. pylori* in response to changes in nickel levels as well as environmental pH,[75,76,363−368] and an HpNikR-deficient strain is not as efficient at colonization in a mouse model.[364] The access of HpNikR to nickel is limited by the urease biosynthetic pathway,[253] even though in vitro the accessory proteins each have a weaker nickel affinity than HpNikR,[159,241,348,350]

providing evidence for intracellular nickel distribution beyond the thermodynamic capabilities of the individual components.

The X-ray crystal structure of apo-HpNikR revealed an asymmetric closed-trans conformation of the tetramer,[343] and upon soaking the crystals with $NiSO_4$, three different pairs of nickel-binding sites were observed that are designated the final site (F), the intermediate site (I) and the external site (X) (Figure 14).[343] Two nickel ions bind on the "locked side" at the square-planar F sites corresponding to the EcNikR high-affinity site (His88′, His99, His101, and Cys107 in the HpNikR numbering). Two nickel ions also bind to the nearby X sites and appear to prevent solvent access to the F sites. The X sites have an octahedral geometry and are composed of His74, His75, Glu104, and a citrate ion from the crystallization buffer. The X site is similar to a second site also observed in PhNikR structures.[336] Two nickel ions on the unlocked side bind to I sites, which contain a mixture of ligands from both the F and X sites.[343] Another X-ray crystal structure obtained from soaking apo-HpNikR crystals with $NiCl_2$ revealed stoichiometric nickel binding with two nickel ions in X sites and two in I sites. The survival of cells expressing HpNikR mutants indicated that all sites contribute to the protective role of HpNikR in response to nickel exposure.[343] On the basis of these structures, it was hypothesized that the nickel could be captured by the X sites of HpNikR and then passed to the F sites via the I site.[343]

Although the crystal structures reveal up to six nickel ions bound to HpNikR, only four ions per tetramer were observed to bind in solution by using metal analysis or titrations monitored by fluorescence or electronic absorption spectroscopy.[348,350,369] Furthermore, the latter method suggests that all four ions are bound to the conserved square-planar site (F),[348] an observation corroborated by XAS analysis (Y.L., D.B.Z., unpublished data). However, sequential nickel binding by four nickel ions into two distinct sites is supported by ITC experiments.[350] In addition, there is no evidence that the presence of more than stoichiometric nickel modulates the affinity of HpNikR for DNA.[348,369−371] Instead, other cations such as Mg^{2+}, Ca^{2+}, Mn^{2+}, Na^+, or K^+ influence the DNA-binding activity of Ni(II)−HpNikR.[369,371] This characteristic may be due to the fact that HpNikR has an extra 9-residue sequence at the N-terminus that is distinct from other NikR homologues, and in particular Asp7 and Asp8 are implicated in the divalent metal requirement.[371]

The *H. pylori* genome seems to contain only a small number of sigma factors and few transcriptional regulators.[372] Only two metalloregulators have been identified, HpNikR and the iron-responsive factor Fur. So it is perhaps not surprising that HpNikR appears to have a lot of responsibilities and works as both a repressor and an activator to control a large variety of genes in response to nickel.[75,76,363,365−367] For instance, HpNikR induces urease but represses NixA expression by binding to the *ureA* and *nixA* promoters in response to nickel.[348,363,366,367,369−371] Genes regulated by HpNikR also include those for other nickel uptake factors (*fecA3*, *frpB4*, and *exbB/exbD*) and nickel storage proteins (*hpn* and *hpn*-like), indicating the critical role of HpNikR in nickel homeostasis.[75] There is also overlap with iron homeostasis pathways because HpNikR controls genes involved in iron uptake and storage (*pfr* and *fur*),[75,373] and Fur binds to the *nikR* promoter.[373] The interaction of Fur and HpNikR with each others' genes suggests an intricate crosstalk between these two global transcription factors in regulating metal homeostasis. Furthermore, HpNikR may also regulate stress response and motility genes.[75] A loose consensus sequence (TATWATT-N_{11}-AATWATA) was determined by scanning mutagenesis of the HpNikR recognition sequence on the *ureA* promoter as well as sequence alignments with several additional target sites,[365,373] but HpNikR can bind to a variety of different DNA sequences.[76,348,365,367,369−371,373,374] Direct comparisons of many of these recognition sequences revealed that they can be roughly divided into two groups based on holo-HpNikR binding affinity, 40−70 nM and 1−10 μM,[374] suggesting that HpNikR can provide two levels of regulation. This property of HpNikR also may be attributed to the extra N-terminal sequence in this homologue, which is important in preventing HpNikR binding to low affinity and nonspecific DNA sequences.[371] How HpNikR can function as both an activator and repressor of transcription is not yet clear. However, activation of the *ureA* promoter occurs upon binding of HpNikR to a site upstream of the promoter, whereas all of the binding sites of the repressed genes overlap the transcription start site,[365] suggesting that in the latter cases HpNikR blocks the RNA polymerase as suggested for EcNikR.[368]

In the stomach, *H. pylori* travels through gastric acid (pH 1−2) before reaching the mucus layer,[375] and it is exposed to pH values ranging from 1 during starvation to 6 during digestion even after initial colonization.[375] Several mechanisms are utilized by *H. pylori* to deal with the variable acidity[368,375−382] and include HpNikR-dependent regulation.[364,368,383,384] HpNikR controls acid-responsive gene expression in *H. pylori* via a repressor cascade that includes Fur and possibly the Hp0166 regulator.[368,383] This response to acid may be due to changes in nickel availability that are sensed by HpNikR, but it is also possible that the regulator itself is influenced by changes in intracellular pH.[385,386] A recent study demonstrated that DNA binding by HpNikR to the *nikR* and *ureA* promoters is distinctly affected by reducing the pH in vitro, suggesting a potential role for HpNikR-regulated gene expression in an acidic environment.[384] However, the detailed mechanism of how HpNikR mediates DNA regulation in response to acid and whether this effect is physiologically relevant is still unknown.

4.2. RcnR

As with other types of metals, the import and export of nickel is finely balanced. The expression of the *E. coli* Ni(II) efflux transporter RcnA (section 2.2) is controlled by the nickel- and/or cobalt-responsive regulator RcnR.[88,89] RcnR belongs to a newly identified regulatory family characterized by a homodimeric antiparallel four-helix bundle, which includes the Cu(I)-responsive *M. tuberculosis* CsoR.[89,387] Apo-RcnR represses transcription by binding to the intergenic region between *rcnA* and *rcnR* with a $K_D \approx 30$ nM, and nickel binding to the protein leads to DNA release and thus derepression.[89]

Sedimentation equilibrium experiments demonstrate that RcnR is a tetramer that is not perturbed by Ni(II) or Co(II),[388] although RcnR may exist as a dimer at much lower protein concentrations. Ni(II) or Co(II) binds to RcnR in a 1:1 stoichiometry (i.e., four metal-binding sites per tetramer), with dissociation constants tighter than 25 and 5 nM, respectively, suggesting that low levels of free nickel and cobalt ions (lower limit $\approx$ 1 nM) in *E. coli* can be detected by RcnR and activate expression of the efflux system that removes the excess ions from the cell.[388] Distinct, but overlapping metal-binding residues were identified in Co(II)-

and Ni(II)-RcnR by XAS and site-directed mutagenesis.[388] The minimal structure was defined as Me(II)-(Cys)(His)$_2$(NH_2-Ser)(N/O)$_2$, among which five ligands (the NH_2-terminus, the His3 amide, and the side chains of His3, Cys35, and His64) coordinate with both Ni(II) and Co(II). The sixth ligand for Co(II) is His60, leaving the identity of the final ligand for Ni(II) unresolved.[388]

Between the two regulators, EcNikR and RcnR provide finely tuned control of nickel homeostasis in *E. coli*. The affinity of RcnR for Ni(II) and the low-affinity EcNikR Ni(II) site are of the same magnitude,[351,388] which results in a smooth transition between complete repression of nickel uptake and activation of nickel efflux, with little room in between for accumulation of excess nickel.[89] This sequential response is also attributed in part to the relative number of the two nickel-activated proteins. Given that only one known EcNikR DNA-binding site exists, and that the low-affinity nickel site is hypothesized to form only when EcNikR is bound to DNA,[338] only one EcNikR needs to be activated per cell. In contrast, the number of RcnR molecules that have to be deactivated by nickel is predicted to be 1−2 orders of magnitude greater based on the range of intracellular concentrations of other repressors in *E. coli*.[388] However, there clearly is some overlap between the systems because inactivating *rcnA* causes a reduction of the basal level of *nik* expression,[388] but the details of how nickel partitions between the different pathways remain to be defined. Finally, RcnR may provide a connection between nickel and iron homeostasis because iron induces *rcnR* expression and RcnR prevents upregulation of *rcnA* in response to iron.[88]

4.3. Nickel Regulators from the ArsR/SmtB Family

M. tuberculosis, another human pathogen that has been studied extensively, infects macrophages and arrests the maturation of their phagosomes.[389] This organism requires a supply of nickel for urease, and a brief mention of a predicted NicT/NixA nickel importer has been made,[390,391] but despite the presence of many metalloregulators, there are no homologues of NikR and the mechanisms of regulation of this importer are unclear. Instead, two *M. tuberculosis* nickel-/cobalt-responsive transcription factors that regulate nickel efflux and belong to the ArsR/SmtB family[392,393] were recently identified, NmtR and KmtR.[391,394]

4.3.1. NmtR

NmtR regulates the *M. tuberculosis nmtA* gene, which encodes a putative P_1-type ATPase efflux pump for Ni(II)/Co(II).[394] NmtR represses *nmtA* expression by binding to the intervening promoter region of the divergent *nmtR* and *nmtA* genes with a dissociation constant around 10 nM at a site predicted to be within a degenerate 12-2-12 inverted repeat sequence.[394] Examination of gene regulation by NmtR revealed that derepression occurs in *M. tuberculosis* when nickel, or to a certain extent cobalt, is supplemented in the growth medium, causing NmtR to release the DNA.[394]

Site-directed mutagenesis and reporter assays were used to demonstrate that six residues in NmtR are obligatory for nickel and cobalt sensing in vivo,[394] and XAS confirmed that, in NmtR, these six residues coordinate with Ni(II)/Co(II) in an octahedral geometry.[395] Sequence comparison between NmtR and the prototypical SmtB revealed that four of the ligands (Asp 91, His 93, His 104, and His 107) are located in the α5 helices and the other two (His 109 and His 116) are in the carboxyl terminus (α5C).[394] One nickel ion per monomer binds dimeric NmtR with $K_D \geq 2 \times 10^7\ M^{-1}$, and Co(II) binds with an affinity ≥40-fold weaker.[395]

Although NmtR does not respond to Zn(II) in vivo, it binds to Zn(II) in vitro with at least 500-fold tighter affinity than to Co(II), implying that the hierarchy of metal binding to NmtR in vitro is Zn > Ni ≫ Co.[394] Zinc is also more tightly bound than nickel or cobalt by the zinc-responsive transcriptional repressor SmtB,[396] and the Zn(II)/Co(II)-responsive CzrA.[395] Given that all three transcription factors bind zinc more tightly than the other metals, it is interesting that Ni(II) and Co(II) rather than Zn(II) influence the DNA-binding activity of NmtR whereas Zn(II) modulates SmtB and CzrA.[394,397,398] This metal selectivity is achieved by the chemical nature of the metals that dictate different coordination preferences. Ni(II)/Co(II) bind to NmtR in an octahedral complex in order to induce DNA release,[394,395] while Zn(II) only associates with a subset of the six ligands and, hence, fails to promote protein−DNA dissociation.[394,395] The selectivity of NmtR in favor of Co(II) and Ni(II) in vivo is achieved by the recruitment of two His ligands from a short C-terminal extension in the protein. This extra sequence is not present in CzrA and SmtB, and instead these proteins are controlled by tetrahedral Zn(II) or Co(II) complexes.[395,396] Experiments with NmtR also reveal that the metal-selective response can be controlled by cytosolic metal availability. Nickel, as the most potent allosteric effector of NmtR in mycobacteria, is totally ineffective when *nmtR* is introduced into a cyanobacterium, where NmtR only responds to cobalt.[394] Metal analysis of each organism indicated that nickel is relatively excluded from the cyanobacterium compared to the mycobacterium.[394]

4.3.2. KmtR

KmtR is the second nickel and cobalt sensor to be characterized in *M. tuberculosis*. Apo-KmtR represses the expression of a putative cation diffusion facilitator (CDF) metal exporter and of itself by binding to a degenerate 13-4-13 dyad symmetry region (5′-CTATTNTNTGCGTNNN-NANGCAGATANNNG-3′) present in both *cdf* and *kmtR* operator-promoters.[391] It is intriguing that two Ni(II)/Co(II) sensors that both regulate efflux systems are present in *M. tuberculosis*. Reporter assays revealed that, in minimal medium, Ni(II) and Co(II) alleviate KmtR-mediated repression in a concentration-dependent manner with maximal expression detected at ∼5 μM Co(II) and ∼30 μM Ni(II).[391] However, in rich medium, KmtR is sensitive enough to be constitutively derepressed by the trace levels of metal present, whereas NmtR is only affected upon metal supplementation.[391] Thus, it appears that KmtR first detects basal levels of cytosolic nickel or cobalt, which are then exported following expression of the CDF transporter. Only when a higher threshold of these metals accumulates does NmtR sense them and allow the expression of the P_1-type ATPase NmtA.[391] These experiments indicate that *M. tuberculosis* depends on two levels of nickel and/or cobalt sensing and supports the significance of these metal ions in this pathogen as well as the ability to respond to metal fluxes.[391]

Consistent with the in vivo data, KmtR has tighter affinities for nickel and cobalt in vitro than NmtR.[391] However, KmtR lacks the conserved amino acids identified as metal-binding ligands in other members of the ArsR/SmtB family. An alignment of several proteins from Actinobacteria that do

not have typical metal-binding sites suggested several potential ligands. Site-directed mutagenesis of His88, Glu101, His102, His110, or His111 revealed that these amino acids are critical for inducer recognition by KmtR, and Asp95, as a potential sixth ligand, is required for repressor function.[391] Although these residues are located around the predicted α5 helix, they differ from the two α5 sites identified in SmtB and NmtR, suggesting that KmtR employs a new sensory site.[391] The distinct locations of the coordination ligands in NmtR and KmtR allow these proteins to regulate gene expression under two levels of surplus cobalt and nickel,[391] an effect that may also be influenced by discrete interactions with the other *M. tuberculosis* nickel and cobalt homeostasis pathways.

4.4. SrnRQ

In the *Streptomyces sp.*, a NiSOD (section 3.3), encoded by *sodN*, is expressed along with an FeSOD encoded by *sodF*. In *Streptomyces griseus*, if nickel is present, then *sodF* is not expressed.[399] The repression of *sodF* is mediated by the proteins encoded by *srnRQ* (*srn* stands for *sodF* repression by nickel), which are downstream of *sodF* and encode two small proteins that form a 4:4 complex with each other.[400] This SrnRQ complex binds to the *sodF* operator at an inverted-repeat sequence, TTGCA-N_7-TGCAA, which overlaps with the transcriptional start site and represses transcription in the presence of nickel.[399,400]

SrnR contains a putative helix-turn-helix DNA-binding motif and exhibits homology with the ArsR family, so it has been assigned the role of DNA binding.[400] The corepressor SrnQ has 26% arginines and binds approximately 1:1 nickel with an apparent K_D of 0.65 μM on its own, with very little binding of other metals detected.[400] Thus, SrnR and SrnQ are proposed to cooperate as a repressor in which SrnR binding to DNA is regulated by the nickel-loaded SrnQ, although the mechanism of this process is not yet clear.[400] To our knowledge, SrnRQ is the first example of a two-component metalloregulator that is nickel-responsive, although there are hints of other nickel-dependent two-component systems,[401−403] and the first two-component member of the ArsR/SmtB family.

4.5. Nur

Similar to *S. griseus*, *Streptomyces coelicolor* produces two types of SODs in order to cope with oxidative stress, NiSOD and FeSOD.[278,404,405] In the presence of nickel, the expression of *sodN* and *sodF* are increased and repressed, respectively, at the transcriptional level.[278,404−406] This divergent control in response to nickel results in a constant level of SOD activity.[405] Because the *sodF* promoter region of *S. coelicolor* contains the dyad-symmetric SrnRQ-binding sequence defined in *S. griseus*, it was initially thought to be regulated in the same manner. However, a homologue of SrnRQ was not found in the *S. coelicolor* genome. Instead, a different nickel-responsive repressor, Nur, was isolated from cell extracts bound to the *sodF* promoter in the presence of nickel.[407,408]

Despite the fact that both Nur and SrnRQ repress the expression of the *sodF* gene in a nickel-responsive manner, they share no similarity in amino acid sequences or putative regulatory mechanisms.[407] Instead, Nur is 27% identical and 48% similar to *E. coli* Fur.[407,408] However, the residues predicted to constitute the regulatory metal site in Fur are poorly conserved in Nur,[407] and Nur is specific for Ni^{2+} both in vitro and in vivo.[407] A recent structure of Nur crystallized in the presence of nickel revealed a dimeric protein with

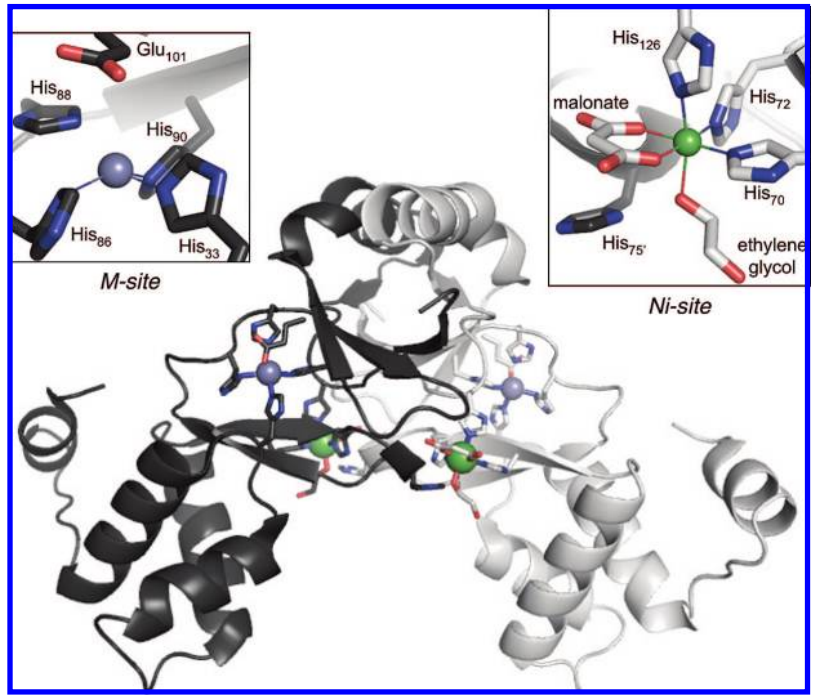

Figure 15. Crystal structure of the *S. coelicolor* metalloregulator Nur. The dimeric protein (pbd 3EYY) is shown with each monomer in a different shade of gray. Two metal sites are observed (insets, sites rotated for clarity). The M-site at the interface of the DNA-binding and dimerization domains is shown here occupied by zinc, with the metal coordinated by His33, His86, and His90. The nitrogen of His88 is ~2.6 Å away from the metal center, so whether it serves as a ligand is not clear. Nickel is also observed in the M-site bound to the same four histidine residues in a square-planar geometry (not shown in this structure). The Ni-site is also at the domain interface and is coordinated to three histidines as well as malonate and ethylene glycol from the crystallization buffer. The color scheme is nickel, green; zinc, gray; oxygen, red; and nitrogen, blue. The pictures were generated by using MacPymol.

two winged-helix DNA-binding motifs bridged by the pair of dimerization domains (Figure 15).[409,410] Furthermore, two types of metal sites were observed at the interface between the two domains. The first site has a low-occupancy nickel bound in a square-planar geometry to four histidine residues. This site was called the "M-site" because it appears that zinc can also bind in an overlapping site, and the spatial location corresponds to that of zinc in a Fur structure.[411] The surface-exposed octahedral "Ni-site" has nickel bound to three histidine residues and two oxygen-containing small molecules. In vitro analysis of mutant proteins revealed that ligands at both metal sites contribute to the metal-responsive DNA-binding activity, so it is likely that they both have regulatory roles in vivo. Given the location of the metal sites at the domain interface, it is feasible that metal binding would influence the orientation of the DNA-binding domains and regulate activity. In addition, the Ni-site, which is unique to the Nur orthologues, appears to influence the secondary structure of the dimerization domain such that it affects the DNA contacts made by the protein in a manner that is distinct from other members of the Fur family.

Holo-Nur binds to the *sodF* promoter in vitro, consistent with the constitutive nickel-independent production of Fe-SOD in a Δ*nur* strain.[407] In contrast, although *sodN* was upregulated in the wild-type strain supplemented with nickel and NiSOD is not expressed in the Δ*nur* strains, neither apo- nor holo-Nur bind to the *sodN* promoter in vitro.[407] These experiments suggest that Nur regulates *sodN* indirectly, possibly by repressing a molecule that serves as the *sodN* repressor, as proposed for Fur from some organisms.[408] In addition to the SODs, Nur also regulates a NikABCDE uptake transporter by binding to the *nikA* promoter and repressing transcription in response to nickel,[407] and it may also regulate expression of a NikMNOQ transporter.[409]

5. Concluding Comments

Over the past few years, significant progress has been made in our knowledge about the pathways that contribute to nickel homeostasis and about the properties of the individual components. These advances include the discovery of outer-membrane transporters and nickel-specific efflux systems as well as tantalizing information about the nickel complexes that are taken up by the cell. Several classes of nickel metalloregulators have been identified, and structural, biochemical, and spectroscopic studies illustrate details about their mechanisms of action. The characterization of many of the factors involved in enzyme metallocenter assembly and/or storage provides information about their metal-binding activities and suggests how they interact with each other. These advances impart a deeper understanding of the nickel−protein complexes as well as their physiological functions. However, there are many aspects of these individual systems that remain unknown, as discussed above. Furthermore, there are some collective issues about intracellular nickel use that are not understood. For example, although we are starting to get some information about the overlap between the control of nickel and other metabolites through the studies of NikR, very little is known about how these separate systems are integrated in the context of the cell. This issue includes whether there is exchange of information between the separate nickel pathways themselves. For instance, is there direct communication between the nickel chaperones and nickel regulators? Moreover, there are questions about the distribution of nickel ions, such as whether the nickel that is brought into the cytoplasm is transferred directly from the transporter to a nickel chaperone? Or, how much nickel is not bound to the dedicated nickel proteins and what form does it take? Answers to these questions will not only afford insight into nickel homeostasis but will also expand the definitions of the principles that govern transition metal homeostasis, a key factor of life.

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7. References

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