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Specific Metal Recognition in Nickel Trafficking

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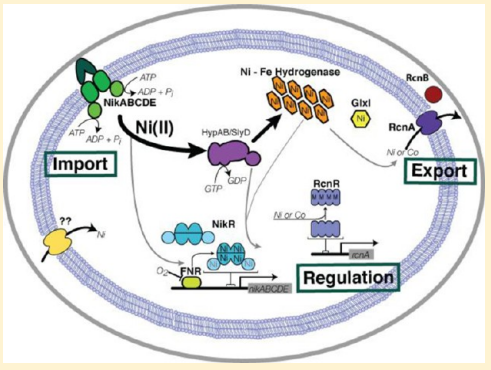
ABSTRACT: Nickel is an essential metal for a number of bacterial species that have developed systems for acquiring, delivering, and incorporating the metal into target enzymes and controlling the levels of nickel in cells to prevent toxic effects. As with other transition metals, these trafficking systems must be able to distinguish between the desired metal and other transition metal ions with similar physical and chemical properties. Because there are few enzymes (targets) that require nickel for activity (e.g., *Escherichia coli* transports nickel for hydrogenases made under anaerobic conditions, and *Helicobacter pylori* requires nickel for hydrogenase and urease that are essential for acid viability), the "traffic pattern" for nickel is relatively simple, and nickel trafficking therefore presents an opportunity to examine a system for the mechanisms that are used to distinguish nickel from other metals. In this review, we describe the details known for examples of uptake permeases, metallochaperones and proteins involved in metallocenter assembly, and nickel metalloregulators. We also illustrate a variety of mechanisms, including molecular recognition in the case of NikA protein and examples of allosteric regulation for HypA, NikR, and RcnR, employed to generate specific biological responses to nickel ions.



Many bacteria require nickel for the production of key enzymes, including ureases, hydrogenases, carbon monoxide dehydrogenases, acetyl-coenzyme A synthases, methyl coenzyme M reductases, some glyoxylases, and a unique superoxide dismutase.[1,2] Furthermore, many pathogenic bacteria, including *Escherichia coli*, *Helicobacter pylori*, *Brucella suis*, *Yersinia* species, *Salmonella*, *Shigella*, and *Mycobacterium tuberculosis*, rely on nickel trafficking systems for their survival and pathogenicity.[1,3] *E. coli* and *H. pylori* both require nickel for NiFe-hydrogenases, and in the case of *H. pylori*, nickel is also required for urease.[4−7] As is the case for other transition metals,[8−10] bacteria that utilize nickel have evolved proteins to facilitate the import and export of nickel and its cellular distribution and incorporation into enzymes in response to the nickel levels in the cell. The proteins involved are collectively termed nickel trafficking proteins. Unlike the trafficking systems employed for more common transition metals such as iron, copper, and zinc,[11] the "traffic pattern" for nickel is relatively simple because bacteria that utilize nickel usually feature only one or two target enzymes. Nonetheless, the nickel trafficking system has all the essential features of any transition metal trafficking system and thus offers an opportunity to develop a global perspective of the system (Figure 1). Many of the key proteins involved in nickel trafficking have been identified in various organisms. These proteins include importers, exporters, chaperones and accessory proteins involved in enzyme metallocenter assembly, storage proteins, and regulators and are summarized in Table 1, which also indicates proteins where structural information is available. To function properly, these proteins require a mechanism that allows for discrimination of the metal being trafficked (the cognate metal) from all other metals (noncognate metals), by finding a way to overcome

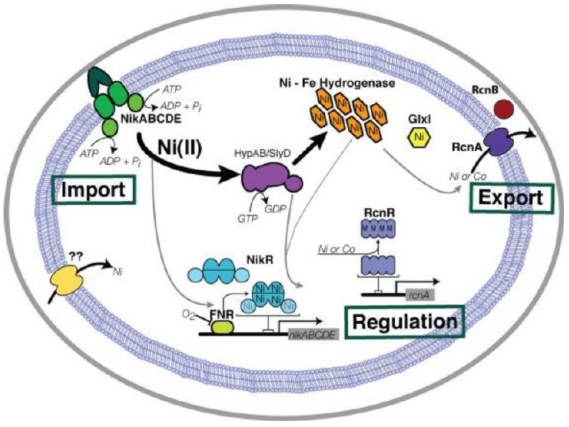


Figure 1. Nickel trafficking in *E. coli* has only one set of target proteins (hydrogenases) but features the required components of all transition metal trafficking systems, an importer (NikABCDE), chaperones and accessory proteins (HypA, HypB and SlyD) used to deliver Ni(II) and incorporate it into hydrogenases, an exporter (RcnAB), and metallosenors that control the transcription of the importer (NikR) and exporter (RcnR).

the binding preferences dictated by the thermodynamics of metal ligand interactions, as reflected in the Irving−Williams series [Co(II) < Ni(II) < Cu(II) > Zn(II)].[108,137,138] The mechanisms employed in specifically binding Ni(II) ions [Ni(II) recognition] are the focus of this Current Topic, and

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Table 1. Proteins Involved in Nickel Trafficking

protein	organism[a]	properties	refs
		Importers	
NikABCDE	*Ec*	ABC-type ATPase; *Ec*NikA is a crystallographically characterized monomeric 56 kDa periplasmic binding protein that transports Ni(L-His)$_2$; metal site XAS; NikB and -C are transmembrane proteins; NikD and E have ATPase activity	12−19
NikZYXWV	*Cj*	an ABC-type transporter that binds solvated nickel with a K_d of 0.61 μM; also binds Co(II)	20
Cbi/NikMNQO	*Se*	a nickel/cobalt permease	21
HoxN	*Ae*	a 33.1 kDa permease located on hydrogenase gene cluster A; Asn31, His62, Asp67, and His68 are critical for permease activity; Cys331 has an important but nonessential function	22−25
NixA	*Hp*	a monomeric 37 kDa permease that is integral in urease maturation; residues His44, Asp49, His50, Asp47, Asp55, Asp194, and Asp231 are essential for nickel uptake, while residues Glu198, Asp234, and Glu274 are important but not essential; residues Phe75, Gly78, His79, Asn127, Trp195, and Ser197 were found to completely abolish nickel uptake and urease maturation; NixA was shown to bind, and possibly import, Co(II), Cu(II), and Zn(II)	26, 27
NhlF	*Rrh*	a NiCoT permease; His74 and His34 are essential for metal transport, while His68 and Phe70 are important but not essential	25, 28
HupN	*Bj*	a 40 kDa nickel permease	29
		Exporters	
CznABC	*Hp*	a cadmium, zinc, and nickel efflux pump; all three genes cause increased sensitivity to cadmium, zinc, and nickel; CznB and CznC both bind nickel; CznABC modulates urease activity; Δ*cznA* and Δ*cznC* cause an 8−10-fold increase in urease activity	30
NreB	*Ax*	nickel efflux pump with a 42-residue C-terminal poly-His tail	31
	Ec	confers Ni(II) resistance and responsible for decreased Ni(II) accumulation levels	31
RcnAB	*Ec*	a nickel and cobalt efflux system whose transcription is controlled by the metalloregulator RcnR; RcnB is a periplasmic protein that does not bind metals	32, 33
MrdH	*Pp*	a 41 kDa nickel, cadmium, and zinc efflux pump	34
NrsD	*S*	a nickel efflux pump with a C-terminal poly-His tail	35
NcrAC	*Lf*	membrane proteins that form a nickel efflux system	36, 37
		Chaperone and Accessory Proteins	
HypA		monomeric or homodimeric metallochaperone that delivers Ni(II) to the apo-H$_2$ase large subunits; contains a structural Zn(II) site that employs highly conserved CXXC motifs; forms heterodimers with HypB and UreE	
	Ec	metal site XAS; binds two Ni(II) and two Zn(II) ions per dimer with micro- and nanomolar affinity, respectively; each monomer is 13.1 kDa	38−40
	Hp	NMR structure; metal site XAS; binds one Ni(II) per monomer (25 kDa) and one Zn(II) per dimer at pH 7.3; at pH 6.3 two of the Zn(II) S donors are replaced by histidines and there is one Ni(II) ion bound per dimer	41−46
	Tk	crystal structure; the 31.4 kDa homodimer engages in a domain swap that involves one CXXC motif from each monomer to form the Zn(II) site	47
	Af	forms a heterodimer with HypB monomer but not HypB dimer; each monomer is 25 kDa	48
HypB		P-loop GTPase required for H$_2$ase maturation and Ni insertion; it is a homodimer that forms heterodimers with HypA and SlyD	
	Ec	a 27.2 kDa (monomer) protein that has two metal binding sites; a high-affinity nickel binding site at the N-terminus and a low-affinity metal-binding site in the GTPase domain that can bind both nickel and zinc; the former has a CXXCGC motif and binds Ni(II) with subpicomolar affinity in a planar site with three S and one N/O donor ligands; the latter site can bind either Ni(II) or Zn(II) with low affinity but prefers Zn(II) over Ni(II); the Zn(II) site is tetrahedral and consists of two Zn(II) ions; binding of a second metal perturbs the metal site structure of the monometalated protein	40, 49−52
	Hp	lacks the high-affinity site found in *Ec*HypB; binds Ni(II) and Zn(II) with a 1:1 stoichiometry with a 1.72 μM affinity; Ni(II) does not affect GTP hydrolysis, but Zn(II) inhibits it; 27.2 kDa monomer	41, 53, 54
	Mj	crystal structure; lacks the high-affinity site found in *Ec*HypB; has two metal binding sites with a dinuclear Zn(II) site, one bound by three Cys residues (Cys95 and Cys127 from one monomer and Cys95 from another) and one H$_2$O and the other by two Cys residues (Cys127 and Cys95), one His (His96), and one H$_2$O; one of the Cys95 residues bridges the Zn(II) ions; 24.3 kDa monomer	55
	Af	crystal structure; a 24.7 kDa monomer that undergoes GTP-dependent dimerization	56
	Bj	possesses 24 N-terminal histidine residues that can bind up to nine Ni(II) ions per monomer (32.8 kDa) with a K_d of 2.3 μM	152, 153

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Table 1. continued

protein	organism[a]	properties	refs
		Chaperone and Accessory Proteins	
	Rl	possesses a polyhistidine tail used for nickel storage or buffering; 32 kDa monomer	154
SlyD		a monomeric peptidyl-prolyl isomerase with chaperone functions involved in the delivery of Ni(II) to [NiFe]-hydrogenases	
	Ec	NMR structures; contains a 50-residue C-terminal tail that binds Ni(II) ions with nanomolar affinity; forms a complex with HypB with a possible role in transferring Ni(II) to HypB; HypB incubated with SlyD shows a 10-fold increase in the level of GTP hydrolysis; 21 kDa monomer	57−74
	Tt	crystal structure; lacks the C-terminal poly-His tail found in *Ec*SlyD; binds one Ni(II) ion per monomer with a K_d of ∼50 nM	75, 76
	Hp	NMR structure; 20 kDa monomer with a 34-residue poly-His C-terminal tail; equilibrium dialysis reveals 2.4 Ni(II) ions per protein with a K_d of 2.74 ± 0.26 μM	77
UreE		metallochaperone that delivers nickel to apo-urease	
	Ka	crystal structures; metal site XAS; binds six Ni(II) ions per 35.2 kDa dimer with a K_d of 9.6 μM; the two essential Ni sites have pseudo-octahedral geometry with six N/O donors, of which three to five are histidines; contains a C-terminal poly-His tail	78−84
	Bp	crystal structures; metal site XAS; lacks the C-terminal poly-His tail of *Ka*UreE; can bind two Ni ions per dimer (41 kDa) with a K_d of 35 μM	85−87
	Hp	crystal structures; metal site XAS; lacks the C-terminal poly-His tail of *Ka*UreE; can bind one Ni or Zn per dimer (38.6 kDa) with a K_d of 0.15 or 0.49 μM, respectively; apo-UreE is a dimer and holo-UreE a tetramer in the solid state	46, 88−92
UreG		P-loop GTPase required for urease maturation	
	Bp	a 46 kDa dimer with a k_{cat} of 0.04 min^{-1}; binds two Zn(II) ions per dimer with a K_d of 42 ± 3 μM and a 10-fold lower affinity for Ni(II)	93
	Mt	a 46.2 kDa dimer with a k_{cat} of 0.01 min^{-1}; held together by a disulfide bond	94
	Hp	metal site XAS; apo-*Hp*UreG is a 22 kDa monomer that binds 0.5 Zn(II) per monomer with a K_d of 0.33 ± 0.03 μM; Zn(II) binding, but not Ni(II) binding (K_d = 10 ± 1 μM), causes dimerization; residues Cys66 and His68 are involved in Zn(II) binding	46, 89, 92, 95
	Ka	apo-*Ka*UreG is a 21.9 kDa monomer that binds one Zn(II) or Ni(II); unlike *Hp*UreG, metal binding does not affect dimerization; residue C72 is involved in Zn(II) binding	96
CooC		a P-loop nickel binding ATPase involved in the incorporation of Ni(II) into carbon monoxide dehydrogenase (CODH); binds to CooJ and CooT (function unknown) in the incorporation of Ni(II) into CODH.	97
	Ch	crystal structures; apo-*Ch*CooC is a monomer (29 kDa) and dimerizes upon ATP binding; metal binding occurs in the dimer interface with a CXC motif from each monomer forming a four-coordinate binding site, involving cysteine residues C112 and C114; this site binds both Ni(II) and Zn(II) with approximately equal affinity	98
	Rr	a 27.8 kDa homodimer that catalyzes ATP and GTP hydrolysis with K_m values 24.4 and 26 μM, respectively	99
CooJ		metallochaperone that is involved in incorporation of Ni(II) into carbon monoxide dehydrogenase	
	Rr	a 12.5 kDa dimer that binds four Ni(II) ions per monomer with a K_d of 4.3 ± 0.3 μM; *Rr*CooJ has a 15-residue histidine-rich tail similar to that seen in *Bj*- and *Rl*HypB, as well as *Ka*UreE	100
		Storage/Ni Buffer Proteins	
Hpn	*Hp*	a 7 kDa, 60-amino acid protein; 28 of them are histidines that form oligomers; most common oligomeric form is a 20-mer; *Hp*Hpn binds five Ni(II) ions per monomer with a K_d of 7.1 μM; Ni(II) binding is reversible in the presence of EDTA or acidic pH (<6.8); the protein contains four cysteines, all of which are involved in metal binding	101, 102
Hpn-like	*Hp*	a 9 kDa, 72-amino acid protein; 18 of them are histidines and 30 glutamines that form oligomers; the most common oligomeric form is a 22(±1)-mer; it binds two Ni(II) ions per monomer with a K_d of 3.8 ± 0.2 μM; Ni(II) binding is reversible in the presence of EDTA or acidic pH (<7.0); it is upregulated at pH 5.0 by ArsRS	103, 104
		Regulators	
NikR		a homotetrameric transcriptional repressor that contains four high-affinity as well as low-affinity metal binding sites	
	Ec	crystal structure; metal site XAS; recognizes the sequence GTATGA-N_{16}-TCATAC within the *nikABCDE* promoter; binds one nickel per monomer (15.1 kDa) with picomolar affinity; the high-affinity binding site binds Ni(II) in a planar geometry using His87, His89, and Cys95 from one subunit and His76 from an adjacent subunit; nickel bound to low-affinity sites increases the affinity for DNA from nanomolar to picomolar; K^+ ions are essential for nickel-responsive DNA binding	105−114

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Table 1. continued

protein	organism[a]	properties	refs
		Regulators	
	Hp	crystal structure; each 17.1 kDa monomer can bind four Ni(II) ions in high-affinity sites with nanomolar affinity; the planar nickel site is similar to *Ec*NikR; ITC suggests *Hp*NikR can bind up to 10 nickel ions in low-affinity sites with micromolar affinity	115−121
	Ph	crystal structure; the high-affinity sites are similar to those in *Ec*NikR; there are four low-affinity sites that are coordinated in a distorted trigonal pyramidal geometry via His64 and Asp65 from one subunit and Asp75 from another subunit	122
	Gu	recognizes GTG(T/C)TAC-X_{13}-GTG(C/T)TAC as the primary DNA sequence and exhibits DNA wrapping	123
RcnR	*Ec*	metal site XAS; 40 kDa tetramer that regulates the expression of the Ni(II)/Co(II) exporters RcnAB; has K_d values of 25 nM (Ni) and 5 nM (Co); binding results in derepression; recognizes a TACT-G_6-N-AGTA DNA sequence; involves Cys35 in binding all metals; cognate metals are distinguished by six-coordinate $(N/O)_5S$ complexation involving the N-terminus; cobalt and nickel are further distinguished by His3, which is a ligand for Co(II) but not Ni(II)	124−127, 170
NmtR	*Mt*	metal site XAS; NMR structure; a 25.6 kDa homodimer that controls the expression of the P-type ATPase NmtA; responds to both Ni(II) and Co(II); binds one nickel per monomer with K_d values of 0.087 and 0.14 nM; residues Asp91, His93, His104, His107, His109, and His116 are necessary for Ni(II) or Co(II) recognition; His3 has been found to be important for cognate vs noncognate distinction; metal site XAS data indicate that the nickel site is octahedral with three His and three other N/O donors	128−130, 172
KmtR	*Mt*	regulates the expression of an exporter protein; Ni(II) and Co(II) binding results in derepression; His88, Glu101, His110, and His111 are required for Ni(II) and Co(II) recognition	131
Nur	*Sc*	crystal structure; represses *sodF* (FeSOD); transcription is activated only in the presence of nickel and occurs even in the presence of EDTA; in the presence of nickel, FeSOD is constitutively expressed; NiSOD expression is abolished; Nur (16.2 kDa) deletion mutants show high levels of accumulation of nickel in the cell	132, 133
SrnRQ	*Sg*	a 99.2 kDa R_4Q_4 octamer that does not require nickel to bind DNA; SrnQ undergoes a conformational change upon binding one nickel	134
NcrB	*Lf*	transcriptional regulator of *pncrA* and *pncrB*; binds to the sequence 5′-ATCCCCCTGGGGGGGAT-3′, and Ni(II) binding leads to derepression	37
Mua	*Hp*	a 36.8 kDa dimer that modulates urease activity by controlling the expression of UreAB without direct DNA binding and imparts nickel resistance; binds one nickel ion per monomer; a secondary regulator; NikR is the primary regulator	135
NimR	*Hi*	regulates the expression the Ni(II) permease NikKLMQO based on cytoplasmic nickel availability and pH; binds one nickel per dimer; is important in the maturation of urease but does not directly control expression of urease genes	136
InrS	*S*	a 44 kDa tetrameric transcriptional regulator that responds to both Cu(I) and Ni(II) in a 1:1 stoichiometry by derepressing the cytosolic exporter *nrsD*; has an estimated K_d of 2.05 pM	137

[a]Abbreviations: *Ae, Alcaligenes eutrophus*; *Af, Archaeoglobus fulgidus*; *Ax, Achromobacter xylosoxidans* 31A; *Bj, Bradyrhizobium japonicum*; *Bp, Bacillus pasteurii*; *Ch, Carboxydothermus hydrogenoformans*; *Cj, Campylobacter jejuni*; *Ec, E. coli*; *Gu, Geobacter uraniireducens*; *Hi, Haemophilus influenza*; *Hp, H. pylori*; *Ka, Klebsiella aerogenes*; *Lf, Leptospirillum ferriphilum* UBK03; *Mj, Methanococcus jannaschii*; *Mt, M. tuberculosis*; *Ph, Pyrococcus horikoshii* OT3; *Pp, Pseudomonas putida* KT2440; *Rl, Rhizobium leguminosarum*; *Rr, Rhodospirillum rubrum*; *Rrh, Rhodococcus rhodochrous*; *S, Synechocystis* PCC 6803; *Sc, Streptomyces coelicolor*; *Se, Salmonella enterica* serovar Typhimurium; *Sg, Streptomyces griseus*; *Tk, Thermococcus kodakaraensis*; *Tt, Thermus thermophilus*.

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by necessity, the focus is on proteins for which knowledge of metal-specific protein interactions and functions is available.

NICKEL IMPORTERS

There appear to be two basic strategies involved in the acquisition of nickel by bacteria. The process can be energetically driven by ATP hydrolysis, as in the case of ABC-type permeases, or by passive transport, as in the case of nickel/cobalt transporters (NiCoTs).

Nik Permease. In *E. coli*, the *nik* operon encodes a nickel ABC-type permease,[139] NikABCDE, that is expressed under anaerobic conditions to satisfy the nickel requirements for NiFe-hydrogenase maturation.[12] Hydrogenases conduct the reversible oxidation of hydrogen to protons and electrons, which can be a source of energy.[4−6,12] The transcription of the *nik* operon is positively regulated under anoxic conditions by the fumarate and nitrate regulatory protein, FNR, a transcription regulator of many genes that are linked to anaerobic and fermentative metabolic functions, and negatively regulated by the binding of nickel to the DNA binding protein NikR.[140−143] The importer is composed of a periplasmic binding protein, NikA, two transmembrane proteins, NikB and NikC, and two ATPases, NikD and NikE.[12,13]

On the basis of the chemistry of other ABC-type transporters, the periplasmic binding protein is likely involved in discriminating nickel from other transition metals. NikA is a 56 kDa monomer that binds Ni(II) ions with a K_d of either of 0.1 or 10 μM.[12,14] NikA can bind other metals, including Fe(II), Co(II), and Cu(II), but the binding of these metals to NikA is at least 10-fold weaker than that of Ni(II).[12] The first structural information regarding the binding of metal to NikA came from X-ray absorption spectroscopy (XAS) data that revealed that the Ni(II) site in NikA is six- or seven-coordinate consisting of six N/O donors at 2.06 Å and a possible S/Cl donor at 2.57 Å.[15]

The first NikA crystal structure was published in 2003[14] and featured Ni(II) ions bound to the protein. The crystal structure revealed that the overall fold of the protein resembled those of other periplasmic binding proteins, which have a clam shell structure, except that in the case of NikA the clam shell was open in the presence of the presumed substrate. The first coordination sphere of the Ni(II) was characterized as being composed of five water molecules, one of which formed a hydrogen bond with the side chain of Arg137, and no direct bonds between the protein and the nickel ion.[14] The distances obtained for the Ni(II)−O bonds in the crystal structure ranged from 2.5 to 3.1 Å (average of 2.8 Å), much greater than that obtained from EXAFS analysis,[14,15] and essentially a nonbonded Ni−O distance. A subsequent crystal structure showed NikA bound to $[Fe(EDTA)H_2O]^-$, with the complex in the same protein locus as the previously characterized Ni(II)-$(H_2O)_5^{2+}$ ion.[16] Isothermal titration calorimetry (ITC) experiments determined that NikA can bind Ni(II) chelated by EDTA with an affinity of 30 μM.[144] The crystallographic data suggested that Ni(II) ions were not the substrate for the transporter but, rather, that some complex, such as that seen in iron−siderophore transport,[16,145] might be required. A third crystal structure of NikA, this time purified from the periplasm in the absence of EDTA, showed the nickel center modeled in a square planar geometry and coordinated by three carboxylate ligands from a compound that was modeled as butane-1,2,4-tricarboxylate (BTC) and a histidine residue, His416.[17] Subsequent mutagenesis studies revealed that His416 is required for nickel uptake.[18] However, no evidence of the presence of BTC in *E. coli* has been found, and attempts to synthesize a Ni(II)−BTC−NikA complex from the apoprotein were unsuccessful.[17] Recent evidence shows that $Ni(H_2O)_6^{2+}$ ions are not transported by NikABCDE but that Ni(L-His)$_2$ is transported.[19] The process is stereospecific, as D-His does not support nickel uptake, and involves cotransport of L-His. This complex is likely recognized by the formation of specific H-bonds in the binding site in a manner similar to the recognition of peptides by the peptide transporter OppA, with which NikA shares a significant degree of amino acid sequence homology (Figure 2).[14,146,147]

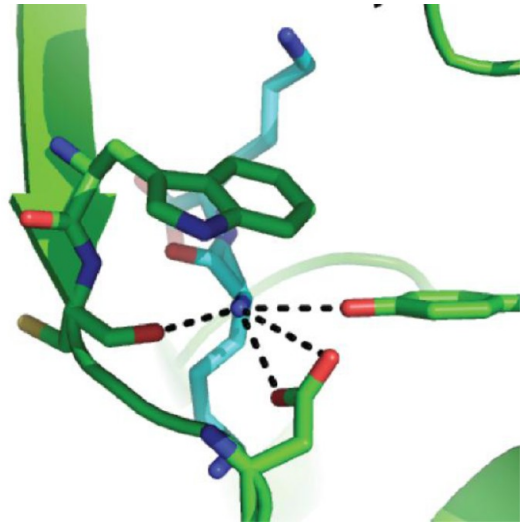

Figure 2. Structure of the peptide transporter OppA with the peptide KK [Protein Data Bank (PDB) entry 2RKM] bound via H-bonds between Lys1 and Tyr109, Cys417, and Asp419.

Although NikABCDE may involve recognition of a specific nickel complex by the periplasmic binding protein, NikA, this mechanism is not universal. Recently, an ABC-type transporter, NikZYXWV, was identified in the human pathogen *Campylobacter jejuni*.[20] Unlike NikA, NikZ does not bind Ni(II) ions chelated by EDTA but binds solvated nickel with a K_d of 0.61 μM. Fluorescence experiments showed that NikL can also bind Co(II) ions.[20]

An additional family of ATP-dependent nickel importers has been found in other bacteria, Cbi/NikMNQO, which like NikABCDE has an ATP binding protein and cytoplasmic components. However, unlike NikABCDE, they lack a periplasmic binding protein.[21]

NiCoT Transporters. NiCoTs make up a family of secondary nickel and cobalt transporters found in prokaryotes and fungi that mediate the uptake of cobalt and nickel ions into the cell.[148]These permeases constitute a distinct structural class of importers that lack ATPase activity.[148] Several NiCoT transporters have been identified in *Alcaligenes eutrophus* (HoxN),[23] *H. pylori* (NixA),[26] *Rhodococcus rhodochrous* J1 (NhlF),[28] and *Bradyrhizobium japonicum* (HupN).[29] NiCoT permeases have an affinity for both Ni(II) and Co(II) ions, as the name implies, and can be divided into three groups: (1) importers specific for Co(II), (2) importers that can transport both Ni(II) and Co(II), and (3) importers specific for Ni(II).[148] Members of this family of permeases feature eight transmembrane helices and conserved Gly-X_5-Glu-His-Ser-Ser-Val-Val and His-X_4-Asp-His sequences.[149] The latter conserved

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motif is associated with nickel binding and is also found in *E. coli* NikC (vide supra).[149] Mutagenesis studies showed that mutations introduced into the His-X_4-Asp-His motif resulted in a decrease in the affinity of HoxN for Ni(II)[24] and NhlF for Ni(II) and Co(II).[25]

NICKEL EXPORTERS

There is virtually no information about the mechanisms of metal recognition in nickel exporters, where characterized proteins include *H. pylori* CznABC,[30] NreB from *Achromobacter xylosoxidans* 31A and *E. coli*,[31] *E. coli* RcnAB (resistance to cobalt and nickel, formerly YohM and YohN),[32,33] *Pseudomonas putida* MdrH,[34] *Synechocystis* PCC 6803 NrsD,[35] and *Leptospirillum ferriphilum* NcrAC.[36,37] Mechanisms similar to those employed by importers may apply to exporters as well, though the delivery of Ni(II) to the exporter may well involve accessory proteins as well as low-molecular weight complexes.

CHAPERONES AND ACCESSORY PROTEINS

The proteins involved in metallocenter assembly in nickel enzymes are a somewhat heterogeneous group from both sequence and functional perspectives. Many examples in which addition of motifs and functions, such as nickel storage, to one accessory protein or another exist. Thus, the discussion of these proteins is dependent on the organism involved. The proteins include HypA, HypB, SlyD, UreE, UreG, CooC, and CooJ, the first four of which are discussed here.

HypA. Every organism that expresses a NiFe-hydrogenase also has a HypA-type nickel metallochaperone to deliver nickel to the apo-H_2ase large subunits. In *E. coli*, HypA and its homologue, HybF, along with HypB and SlyD are responsible for the delivery and incorporation of nickel into three hydrogenases.[38,150] *E. coli* HypA binds two Ni(II) ions per dimer with micromolar affinity[39,41,42] and is copurified with one Zn(II) per monomer that binds with nanomolar affinity to two rigorously conserved CXXC motifs. UV−vis studies showed that HypA can also bind Co(II) to the zinc site.[39]

Like *E. coli* HypA, *H. pylori* HypA binds Zn(II) and Ni(II) ions, exists in solution as a homodimer, and has been shown to form a heterodimeric complex with HypB.[39,41] Cross-linking studies determined that HypA and UreE (vide infra), a urease nickel chaperone, are also capable of forming a heterodimeric complex.[43] Of the five histidine residues present in *H. pylori* HypA, only His2 was shown to be vital for nickel binding.[41] Differential scanning calorimetry as well as CD thermal melts determined that HypA is stabilized by Ni(II) binding, as the binding of Ni(II) to Zn(II)-loaded HypA resulted in an ~12 °C increase in the melting temperature measured using both techniques.[42]

Three structures of HypA proteins have been reported. The first is an NMR structure of the monomeric protein from *H. pylori* (Figure 3),[44] where the N-terminus was modified with additional Gly and Ser residues left after thrombin cleavage of a His tag, and the other two are crystal structures of monomeric and homodimeric forms from *Thermococcus kodakaraensis*.[47] The NMR structure of the *H. pylori* HypA monomer reveals that the Ni(II) site is located at the N-terminus and is coordinated by the backbone nitrogen from His2, Glu3, and Asp40 and the imidazole from His2. The use of His2 as a ligand is in agreement with previous studies.[41] Other NMR work conducted on an unmodified *H. pylori* HypA homodimeric protein showed that there was dipolar broadening in the

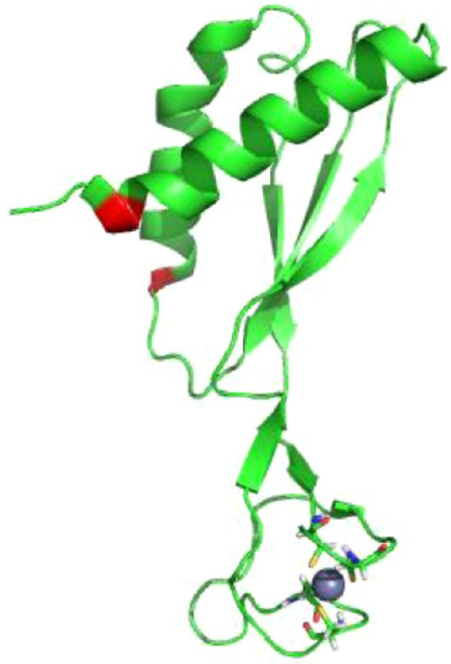

Figure 3. NMR structure of *H. pylori* HypA (PDB entry 2KDX) showing the location of residues involved in nickel coordination: His2, Glu3, and Asp40 (red). The zinc site is shown at the base of the protein and features fours S donor ligands from Cys74, Cys77, Cys91, and Cys94.

N-terminal region (proposed nickel site) upon nickel binding, consistent with a paramagnetic (S = 1) five- or six-coordinate nickel site.[42] It is unclear whether the difference in nickel coordination observed is due to the oligomeric state of the protein or/and the modification of the N-terminal nickel binding site in the monomer structure. The crystal structures of the monomeric and dimeric HypA proteins from *T. kodakaraensis* do not contain Ni(II) ions but do reveal a domain swap in the homodimeric protein that affects the cysteine coordination of the zinc site (Figure 4).[47] All three

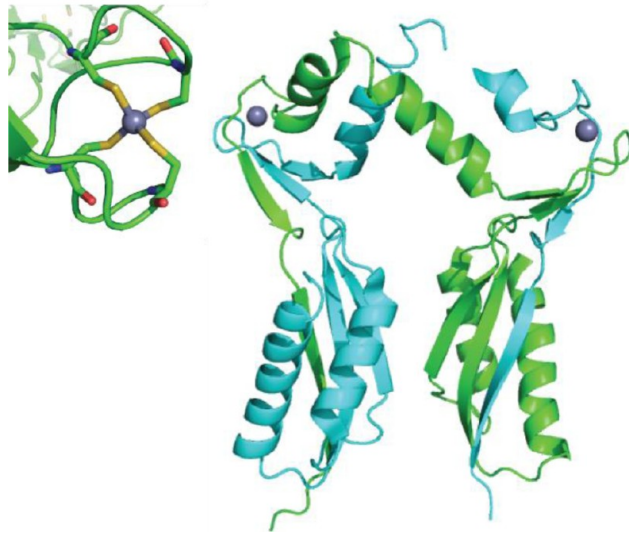

Figure 4. Crystal structure of the zinc site in the monomeric *T. kodakaraensis* HypA protein (left, PDB entry 3A43) and the protein fold in the dimer illustrating the domain swap (right, PDB entry 3A44).

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HypA structures reveal that the zinc is coordinated by the two conserved CXXC motifs. However, in the dimeric form of *T. kodakaraensis*, the Zn(II) ion is coordinated by one CXXC motif from each subunit.

XAS studies of the dimeric form of *H. pylori* HypA showed that the unique structural flexibility of the zinc site goes even further, as it appears to be involved in sensing both Ni(II) binding and pH.[42,45] At pH 7.2, the zinc site in the holoprotein [Ni(II) is bound] is tetrahedral with four S donor ligands.[42] However, a decrease in the pH to 6.3 (the internal pH of *H. pylori* under acid shock) results in a ligand substitution involving the replacement of two S donor ligands on the zinc with two imidazole ligands from histidine residues.[42] This structural change could not be replicated by substituting Co(II) for Ni(II) ions[45] and so appears to reflect a protein conformational flexibility that is metal-specific and may be part of the nickel recognition mechanism. ITC showed that this structural change resulted in a change in the nickel binding stoichiometry from one Ni(II) per monomer at pH 7.2 to one nickel per dimer at pH 6.3. The extent of urease activation increases under acidic conditions,[151] leading to the suggestion that the changes observed in the HypA zinc site monitor protein conformational changes that facilitate the incorporation of nickel into urease under acid stress.[42] One possibility for the interaction of HypA with the urease metallocenter assembly pathway is suggested by a recent study that shows that HypA competes with UreG for binding UreE.[46]

Further studies were conducted using XAS and mutagenesis to probe the connection between the structural changes in the zinc site and the properties of *H. pylori* HypA.[42] In a series of Cys → Ala or Cys → Asp mutations, alteration of any one of the four cysteines resulted in zinc sites that featured a $Zn(Cys)_2(His)_2$ coordination environment, bound only one Ni(II) ion per dimer, and no longer sensed nickel binding or pH changes.[42] The data are consistent with the Cys mutations locking the protein into a conformation that is adopted by wild-type (WT) HypA at low pH. Both of the two CXXC motifs in HypA are flanked by a histidine residue (His79 and His95), a feature unique to *H. pylori* HypA. Mutating either of these residues to Ala also resulted in a protein that did not sense nickel or pH but did result in the zinc site resembling that of the WT HypA zinc site at pH 7.2 (four S donor ligands). The data from the H79A and H95A mutations suggest that these two histidine residues are ligands in the low-pH WT HypA structure and that the mutation of these residues locks the protein in the conformation adopted by WT HypA at neutral pH.

HypB. HypB is a P-loop GTPase required for hydrogenase maturation and Ni(II) insertion.[41,49,50] *E. coli*, *H. pylori*, and *Methanococcus jannaschii* HypB proteins exist as homodimers in solution.[41,50,55] HypB also makes heterodimers with HypA[41] and SlyD (vide infra), and the HypAB dimers have been shown to exist in vivo.[53] The details of the interaction between the *H. pylori* versions of HypA and HypB have been determined by NMR.[53]

In the reduced form, *E. coli* HypB binds two metal ions per monomer.[51] The two metal binding sites are located near the N-terminus and in the GTPase domain of the protein. The CXXCGC motif located near the N-terminus of the protein is associated with the high-affinity Ni(II) site that has a K_d in the subpicomolar range.[51] A low-affinity metal binding site located in the GTPase domain can bind either nickel or zinc but has a higher affinity for zinc than for nickel.[51,52] The GTPase activity measured is low for *E. coli* HypB, with a k_{cat} of 0.17 min^{-1} and a K_m of 4 μM.[50]

XAS studies conducted on *E. coli* HypB revealed that metal binding status is communicated between the two metal sites. XANES analysis determined that nickel binds to the high-affinity site in a four-coordinate planar geometry, and the binding of zinc to the low-affinity site results in a less intense $1s \rightarrow 4p_z$ transition and a slightly more intense peak at 8442 eV (white line) that indicates a small distortion in the planar geometry and a reduction in the S donor ligand content of the nickel site.[52] EXAFS analysis reveals that the nickel site is composed of three S donors at 2.17 Å and one N/O donor at 1.87 Å. When zinc binds in the low-affinity site, there is a decrease in the Ni−S bond distance to 2.15 Å and an increase in the Ni−N bond distance to 2.02 Å.[52]

Like the nickel site, the zinc site in the G domain is affected by nickel binding to the high-affinity site. XANES analysis revealed that the zinc site is four-coordinate and tetrahedral both with and without nickel in the high-affinity site.[52] However, EXAFS analysis revealed that in the absence of nickel, the zinc site contains two Zn(II) ions with an average of 2.5 S donors, one O donor, one imidazole ligand, and a Zn−Zn interaction.[52] When nickel binds to the high-affinity site, the zinc site becomes more symmetric with an average of two S donors, one N donor, one imidazole ligand, and a Zn−Zn interaction.[52] These subtle structural perturbations suggest an allosteric recognition mechanism like that found in HypA may operate in HypB as well.

The *H. pylori* HypB protein lacks the N-terminal high-affinity nickel site and binds Ni(II) ions in a 1:1 stoichiometry with a 1.72 μM affinity.[53] The protein can bind both nickel and zinc, but zinc competes with nickel for binding, suggesting that the nickel and zinc sites have ligands in common.[54] Mutagenesis studies revealed that Cys106 and His107 located in the G domain are essential for metal binding, as mutating these residues resulted in a protein that could no longer bind nickel and lowered the affinity for zinc by 2 orders of magnitude.[54] Like that by *E. coli* HypB, GTP hydrolysis by *H. pylori* HypB is slow. Studies with *H. pylori* HypB determined that nickel binding, which causes dimerization, does not affect GTP hydrolysis whereas zinc binding, which does not promote dimerization, inhibits HypB GTPase activity.[54] In contrast, other studies have indicated that monomeric HypB is in a low-activity state, and that dimerization to a more active form occurs upon binding GTP or nickel.[53] Binding of Ni(II) ions was proposed to form a "metallic bridge" that stabilizes the active dimer, in which two GTP hydrolysis active sites are formed by the invariant Lys168 residues.[53] However, one study provides data that indicate that apo-HypB (monomer) and the Ni(II) complex (dimer) have approximately the same activity (k_{cat}/K_M),[54] while the other indicates that the dimeric complex with Ni(II) is more active.[53] The origin of these discrepancies is not known.

HypB from *M. jannaschii* also lacks the N-terminal high-affinity nickel site found in the *E. coli* protein. The crystal structure of HypB from *M. jannaschii* (Figure 5) revealed the presence of two metal binding sites involving residues in the G domain, including a dinuclear zinc site located at the dimer interface, where one zinc is coordinated by three Cys residues (Cys95 and Cys127 from one monomer and Cys95 from the other) and a water molecule, and the other Zn(II) is coordinated by two Cys residues (Cys95 and Cys127), one

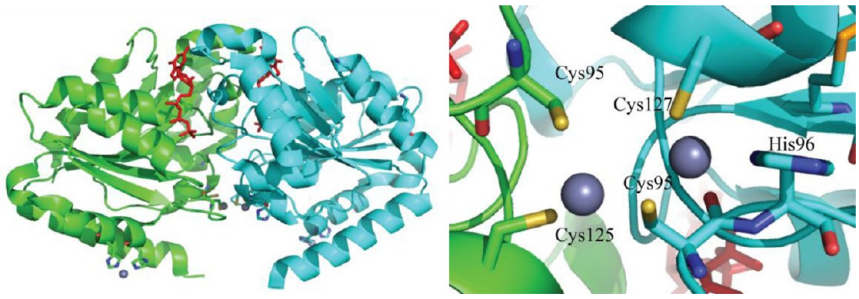

Figure 5. Crystal structure of *M. jannaschii* HypB (PDB entry 2HF8). The left panel shows the HypB dimer with two GTP molecules (red) and two Zn(II) atoms (slate) bound at the dimer interface as well as two Zn(II) atoms (slate) bound at the surface of each monomer. The right panel is a close-up of the zinc site at the dimer interface.

His (His96), and a water molecule.[55] The zinc sites are bridged by a cysteine residue (Cys95).[55] The structure also revealed that HypB binds guanosine 5*-*O*-(3-thiotriphosphate) (GTPγS) at the dimer interface using residues from both monomers.[55]

HypB proteins from *B. japonicum* and *Rhizobium leguminosarum* feature a histidine rich tail[152−154] that plays a role in nickel storage or buffering.[153,155] *B. japonicum* HypB has 24 N-terminal His residues that can bind up to nine nickels per monomer with a K_d of 2.3 μM.[152] The protein can also bind other metals, including Zn(II), Cu(II), Co(II), Cd(II), and Mn(II).[152] Similar polyhistidine regions have been observed in SlyD (vide infra) and other accessory proteins involved in the maturation of urease and carbon monoxide dehydrogenase biosynthesis, UreE (vide infra) and CooJ, respectively.[78,88,100]

SlyD. SlyD (sensitive to *lysis D*)[57] was originally identified as a protein that sensitizes *E. coli* to lysis by bacteriophage φX174.[58,59] SlyD orthologs are found in many species such as *H. pylori*, *T. thermophilus*, *Methanococcus thermolithotrophicus*, and *Vibrio cholera*.[60,61,75,77,156] NMR solution structures of *E. coli* SlyD reveas two domains:[60,62,75] a peptidyl-prolyl isomerase (PPIase) domain and the "inserted in the flap" (IF) domain. The PPIase domain is related to the FK506-binding protein (FKBP)[57,58,157,158] family of peptidyl-prolyl isomerases and catalyzes the *cis*−*trans* isomerization of proline peptide bonds,[62−64,159] which is a crucial step in the folding pathway of some proteins.[160−162] However, the isomerase function of SlyD has been shown to be nonessential in the role of SlyD in hydrogenase maturation.[64,65] Although loading metal ions into the metal-binding domain is required for hydrogenase assembly,[65−67] it has also been shown to disrupt PPIase activity.[64] This is consistent with current data that suggest that metal-loaded SlyD exists in a closed conformation.[64,65,67−70] The IF domain is so termed because it is inserted into the flap region of the FKBP domain.[68,71,72,159] This insertion is observed in a small subset of FKBPs,[62,158] is associated with the chaperone function, and is involved in protein folding.[61,62,68,71,159,163]

E. coli SlyD has an unstructured 50-residue C-terminal tail, termed the metal-binding domain (MBD), which contains 28 potential metal-binding residues (15 His, 6 Cys, and 7 Asp/Glu residues)[57,63,66,70] that bind a variety of first-row transition metals with affinities that follow the Irving−Williams series, but its function is specific to nickel binding in vivo.[70] This tail is variable among SlyDs from different organisms[60,61,75,164] and allows SlyD to bind multiple metal ions with nanomolar affinity, although the exact number of metal ions has not been definitively determined.[60,63,64,66,69,70] SlyD participates in hydrogenase maturation through a complex formed with HypB (vide supra) via the SlyD IF domain.[62,66,67,73,155] The HypB−SlyD complex forms in the absence of HypA (vide supra) or hydrogenase, and heterodimer formation is disrupted by mutation of the two conserved proline residues in HypB.[67] Truncation of the MBD of SlyD or mutations in either of the two metal-binding domains in HypB result in a reduced level of hydrogenase maturation.[52,67] *E. coli* HypB lacks the histidine rich N-terminal region found in other HypB proteins that can bind multiple nickel ions with high capacity but low affinity,[150,152−155] and this lack of a nickel storage or buffer capacity in *E. coli* HypB is complemented by the metal-binding C-terminal extension found in *E. coli* SlyD.[63,64,74] This modular model is supported by the observation that Δ*slyD* strains have reduced cytoplasmic nickel content and hydrogenase activity compared to those of the wild type.[65,66,74] The ability of the metal-binding domain of SlyD to bind multiple metal ions has also been postulated as the source for Ni(II) ions during hydrogenase active site assembly.[63,66,74]

It is thought that one of the roles of the SlyD is to enhance the release of Ni(II) from the high-affinity site of HypB,[51,65−67,73] which binds Ni(II) very tightly [$K_d = (1.3 \pm 0.2) \times 10^{-13}$] and may require some structural change to facilitate the release of the Ni(II) ions from the high-affinity sites.[51] Additionally, studies have shown that the addition of SlyD to HypB with Ni(II) loaded in the high-affinity site results in a 3-fold increase in the catalytic efficiency of GTP hydrolysis.[66] Addition of SlyD to HypB with both the high-affinity and low-affinity sites occupied also increases the catalytic efficiency of GTP hydrolysis by 10-fold.[66] This effect has been traced to the transfer of metal from the low-affinity HypB site to the metal-binding domain of SlyD.[66]

UreE. Synthesis of the urease active site is a complex and highly choreographed process involving several accessory proteins, including UreD, UreE, UreF, and UreG. Little is known about UreD, but it has been suggested that this is the first protein that binds to apo-urease.[165] UreE is the chaperone that delivers nickel to urease.[1,165] UreF has been suggested to be an activator of the GTPase activity of UreG.[94,166]

UreE from *Klebsiella aerogenes* binds six nickels per dimer with a K_d of ∼9.6 μM.[78] X-ray absorption spectroscopy and magnetic circular dichroism spectroscopies suggest that the nickel sites have pseudo-octahedral geometry with six N/O donor ligands, of which three to five are histidines.[78] The stretch of the last 15 amino acids at the carboxyl terminus includes 10 histidine residues.[79] Similar to the situation in HypB (histidine rich region located at the N-terminus) and SlyD (histidine rich region located at the C-terminus) proteins, this polyhistidine C-terminus is not a universal feature of UreE

orthologues and is not found in either *Bacillus pasteurii* UreE or *H. pylori* UreE. *H. pylori* UreE is capable of binding only one Ni(II) and Zn(II) ion per dimer with K_d values of 0.15 and 0.49 μM, respectively.[88,89] *B. pasteurii* UreE binds two Ni(II) ions per dimer with a K_d of 35 μM.[85] NMR and XAS spectroscopies revealed that the nickel sites in *B. pasteurii* UreE are also six-coordinate and involve histidine ligands.[85,86]

The lack of importance of the UreE polyhistidine tail for the incorporation of nickel into apo-urease was also demonstrated using a truncated UreE from *K. aerogenes*, H144*, where the last 15 residues (including 10 His residues) had been removed. This truncated protein was shown to reduce the number of nickel ions bound to approximately two Ni(II) per dimer,[80] like that for the *B. pasteurii* protein, and its ability to incorporate nickel into urease was unaffected.[80]

Other metals, including Cd(II), Co(II), Zn(II), and Cu(II), were shown to compete with Ni(II) for the nickel binding site.[80] These metals coordinate UreE with distinct coordination geometries, as revealed by a number of spectroscopic techniques that were used to characterize the various coordination environments of the divalent metals bound to H144* UreE. The two Ni(II) sites were found to be pseudo-octahedral with six N/O donor ligands, are structurally similar to those found in the WT UreE protein, and are distinguished by different numbers of His ligands.[81] Mutagenesis revealed that His96, His112, and Asp111 are involved in binding Ni(II) in one of the binding sites, while H110 is involved in the second site.[82] In contrast, the two copper sites are tetragonal (CN = 4) with two His ligands each. Additionally, one copper site involved a Cys ligand[81] that was shown by mutagenesis to be Cys79 in H144* UreE.[82] Like the nickel sites, the cobalt sites have $(N/O)_6$ pseudo-octahedral coordination and different numbers of His ligands. His96 and His110 were also identified as ligands to Co(II). These data show that different metals are bound to UreE using different coordination numbers and different ligands, and thus, the coordination geometry and ligand selection of the bound metal provide a mechanism for metal recognition that is similar to that employed by the metalloregulators NikR and RcnR (vide infra).

There are several UreE crystal structures available: (1) *K. aerogenes* (H144*) UreE with copper(II) bound,[83] (2) *B. pasteurii* UreE with zinc bound,[87] (3) *H. pylori* UreE in three forms [apo form and complexes with copper(II) or nickel],[90] and (4) *H. pylori* UreE in three forms (apo form and complexes with zinc or nickel).[91] The *K. aerogenes* (H144*) UreE bound three copper(II) ions in three metal sites per dimer. One copper(II) site was located at the dimer interface and was coordinated by His96 from the two subunits, while the copper(II) ions in the other two sites were coordinated by His112 and His110. All three of these histidine residues were previously identified as nickel binding residues (vide supra).[82] Similar to the first metal-binding site in *K. aerogenes*, the zinc-bound UreE crystal structure from *B. pasteurii* revealed that zinc was coordinated in a tetrahedral fashion by four conserved His100 residues, one from each subunit in a tetramer.[87] The histidine residues coordinated to the metals at the dimer and tetrameric interface of UreE from *K. aerogenes* (His96) and *B. pasteurii* (His100), respectively, are conserved in all UreE proteins and are important for urease activation in vitro[84] and in vivo.[82]

The crystal structures of *H. pylori* UreE were determined for the apoprotein as well as complexes with Cu(II) or Ni(II).[90] The structures showed that the apoprotein formed dimers, while the metal-bound protein was tetrameric (dimer of dimers), with the tetramer formed by coordination of the metal ion by His104 residues from each subunit. A second set of *H. pylori* UreE crystal structures were obtained for the apoprotein and zinc or nickel complexes[91] and implicated the disordered C-terminus in metal binding as well as stabilizing interactions with UreG in the case of the zinc complex.[89] These crystal structures revealed that the nickel site is six-coordinate and interacts with His102 from one monomer and His102, His152, and Glu4 from another, as well as a water molecule and one other unknown ligand.[91] In contrast, zinc is coordinated in a tetrahedral geometry by pairs of His102 and His152 from each monomer.[91] These metal sites were also interrogated by XAS, which confirmed the respective metal geometries and the use of His152 residues that are disordered in the crystal structure.[91] Thus, it was shown that the metal ion selectivity of UreE is based on the different metal ion coordination environments that are dictated by the electronic properties of the metal ion in a mechanism that is facilitated by the flexibility of the C-terminal protein region. The cross-talk among UreE, nickel, and zinc suggested a specific functional role for different metal complexes of this UreE protein in regulating the formation of protein−protein complexes involved in enzyme maturation and pointed to a mechanism for nickel transfer involving the H152 ligands, which could be replaced by UreG residues, leading to the transfer of nickel from UreE to UreG.

METALLOREGULATORS

Metalloregulators control the expression of metallotransporters in response to the cellular metal status. Bacterial metalloregulators acquire metals in a process that involves metal binding affinities, access to metals in the cytosol, and protein allostery.[10,138] Several Ni(II)-responsive metalloregulators have been identified, including NikR,[140] RcnR,[124] NmtR,[128] KmtR,[131] Nur,[132] SrnRQ,[134] NcrB,[37] Mua,[135] NimR,[136] and InrS.[137] However, information regarding Ni(II) recognition is available for NikR, RcnR, and Nur and is discussed in detail below.

To control the level of nickel in the cell, *E. coli* utilizes two transcriptional regulators, NikR (also found in a number of bacteria, including *H. pylori*)[115,122,123,140] and RcnR (vide infra).[124] The first regulator, NikR, controls the expression of the ABC-type nickel importer, NikABCDE,[140] and the second, RcnR, regulates the expression of the exporter at higher metal levels, similar to that seen for the zinc-responsive regulatory proteins, Zur and ZntR in *E. coli*.[167] In *H. pylori*, NikR also controls the expression of a nickel importer, NixA, a NiCoT permease, as well as a variety of other genes, including the urease structural genes (*ureA* and *ureB*), nickel uptake factors (*fecA3*, *frpB4*, and *exbB*/*exbD*), nickel storage genes (*hpn* and *hpn*-like), and genes associated with iron uptake (*fur* and *pfr*).[116]

NikR. NikR is the only member of the ribbon−helix−helix (β−α−α) family of prokaryotic DNA-binding proteins for which the function is regulated by metals.[105] Nickelated NikR binds to a 28 bp palindromic operator within the NikABCDE promoter, GTATGA-N_{16}-TCATAC, with nanomolar affinity.[106,107] *E. coli* NikR is a homotetramer that binds one nickel ion per monomer, and competition assays determined that NikR binds Ni(II) with picomolar affinity.[106,108] *E. coli* NikR (Figure 6) has two distinct metal binding sites, the "high-affinity" site located at the tetramer interface near the C-terminus and the "low-affinity" site, which is suggested to be

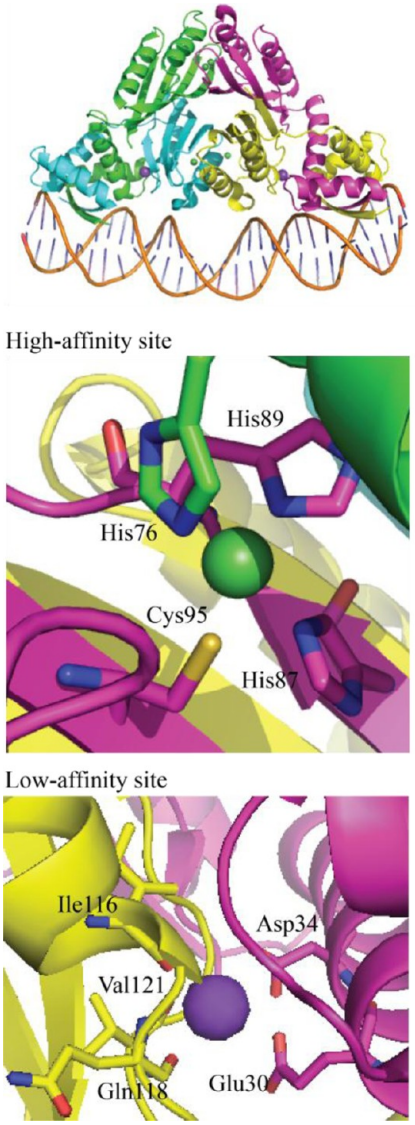

Figure 6. *E. coli* NikR crystal structure (top) showing the location of the high-affinity sites with nickel (green) bound and the low-affinity sites with K^+ ions (lavender) bound (PDB entry 2HZV). High-affinity nickel site (middle) with nickel coordinated by the side chains of His87, His89, and Cys95 from one subunit and His76 from another. Low-affinity site (bottom) with K^+ coordinated by the side chains of Glu30 and Asp34 from the DNA-binding domain and by the carbonyl oxygens of Ile116, Gln118, and Val121 from the metal-binding domain.

located near the interface of the C-terminal domain and the DNA-binding domain.[106,107,122] In the high-affinity site, the nickel ions are bound in a four-coordinate planar geometry by the side chains of His87, His89, and Cys95 from one NikR monomer and by His76 from an adjacent monomer.[109] In addition to these residues, there are three other potential ligands (Tyr60, His62, and Glu97) near the high-affinity Ni(II) site.[110] NikR can bind a variety of other transition metals in vitro, and the binding affinities follow the Irving–Williams series [Co(II) < Ni(II) < Cu(II) > Zn(II)], indicating that there is no unusual affinity leading to a preference for metal binding.[108] Nonetheless, the protein responds in vivo only to the presence of Ni(II) ions.[110] Additionally, the nickel-loaded NikR protein was less susceptible to chemical and thermal denaturation.[108]

Studies of the metal site structure using XAS showed that each metal adopts a distinct structure.[110] The noncognate metals Co(II), Cu(I), Cu(II), and Zn(II) adopt a variety of alternate geometries and ligands.[110] Cobalt binds to the protein, forming a six-coordinate complex with N/O donor ligands, of which three are imidazoles from histidine ligands. Unlike the nickel site, the cobalt site was not coordinated by the S donor ligand, Cys95. Similar to the nickel site, the copper(II) site was found to be four-coordinate planar with three N/O donors, of which two were imidazole and a S donor at 2.21(2) Å, ~0.08 Å longer than the Ni–S bond distance [2.13(2) Å]. Additionally the copper(II) site featured one fewer imidazole ligand than the Ni(II) site. Copper(I) and zinc form three-coordinate trigonal and four-coordinate tetrahedral complexes, respectively. These results suggest that metal ion selectivity in NikR is achieved by the coordination number and/or geometry of the metal–protein complex, as well as ligand selection,[110] which could be coupled to protein allosteric changes, an emerging theme in metal recognition by metalloregulators.[9]

NikR has other putative nickel binding sites, known as the low-affinity sites that bind nickel or other ions.[109−111,117] DNA binding studies demonstrated that nickel binding to a set of low-affinity sites resulted in an increase in the protein's affinity for DNA.[106] Further studies showed that the affinity of NikR for DNA increased from nanomolar to picomolar in the presence of excess nickel.[107] XAS studies performed on heterometalated samples with copper(II) loaded into the high-affinity site and nickel in the low-affinity site reveal that nickel adopts an octahedral geometry with nickel coordinated by two histidine ligands at 1.97(2) Å and four N/O donors at 2.11(2) Å.[110] Furthermore, mass spectrometry and mutagenesis studies identified two histidine residues, His48 and His119, located at the interface of the metal- and DNA-binding domains that participate in the binding of Ni(II) to the low-affinity site.[112] Crystallography experiments performed on *E. coli* NikR crystals soaked with excess $NiCl_2$ in the absence and presence of DNA identified six potential low-affinity Ni(II) sites on the surface of the protein.[113] Most of the sites identified were six-coordinate with N/O donor ligands and at least one histidine residue coordinated to nickel. Additional studies revealed that K^+ ions are essential for *E. coli* NikR nickel-responsive DNA binding.[111]

Like *E. coli* NikR, *H. pylori* NikR binds four Ni(II) ions in the high-affinity sites with nanomolar affinity[115] in a four-coordinate planar geometry coordinated by the side chains of the corresponding residues, His99, His101, and Cys107 from one metal-binding domain and His88 from another.[118] The difference in binding affinity measured in *E. coli* and *H. pylori* NikR may be a due to the differences in the methods as well as the assay conditions used to determine the affinities, a competition assay versus ITC.[168] ITC experiments determined that *H. pylori* NikR can bind up to 10 nickel atoms in the low-affinity sites with micromolar affinity.[115]

The crystal structures of the NikR protein from *E. coli*,[109,114] *Pyrococcus horikoshii*,[122] and *H. pylori*[119] show that the NikR protein can adopt a variety of conformations that might represent protein allostery that could be coupled to metal ion selection. An open conformation was observed in the *E. coli* and *P. horikoshii* apo-NikR crystal structures where the DNA-binding domains are linearly placed on each side of the transmembrane domain.[114,122] A closed *trans* conformation was observed for nickel-bound *P. horikoshii* and *H. pylori* NikRs, where the DNA-binding domains are on opposite sides of the transmembrane domain.[119,122] The nickel-bound *E. coli* NikR–DNA structure

shows a closed *cis* conformation in which the two DNA-binding domains are located on the same side of the transmembrane domain and are bound to DNA.[109]

The evidence coupling these conformations to metal selectivity is less clear. Data from small-angle X-ray scattering (SAXS) experiments did not support these large conformational changes upon binding of nickel to *H. pylori* NikR, as the apoprotein and nickelated protein had very similar scattering patterns.[120] Changes in protein dynamics have been proposed on the basis of computational and NMR studies that suggest that NikR in solution is interconverting among the *cis*, open, and *trans* forms, and that the binding of nickel facilitates the interconversion.[169]

RcnR. RcnR is a member of the recently characterized CsoR/RcnR family of transcriptional regulators that regulates the expression of the nickel and cobalt exporter, RcnA, and the associated periplasmic protein, RcnB.[33,124] RcnR is a tetrameric α-helical protein that binds to a variety of metals but responds only by releasing DNA upon binding nickel and cobalt, which have K_d values of 25 and 5 nM, respectively.[125] The binding of nickel and cobalt is believed to result in a conformational change in the protein that disfavors the binding of DNA, resulting in the expression of the nickel and cobalt exporter, RcnAB.[32,124] It has been shown that apo-RcnR recognizes a TACT-G_6-N-AGTA sequence, two of which are located in the *rcnA*−*rcnR* intergenic region.[126,170] Additionally, RcnR interacts with flanking DNA regions (~50 bp), leading to DNA wrapping.[170] XAS data show that in contrast to NikR, RcnR forms six-coordinate complexes with its cognate metal ions [Ni(II) and Co(II)] and four-coordinate complexes with noncognate metals.[125,127] Both cognate metals adopt an $(N/O)_5S$ ligand environment that involves coordination of Cys35, the only Cys residue in the protein.[125,127] The sites binding the cognate metals differ in M−S bond distances as well as the number of imidazole ligands. XAS studies determined that the Co−S bond distance is 2.31(2) Å, whereas the Ni−S bond distance is 2.62(2) Å.[127] Additionally, cobalt binds one more imidazole ligand than nickel (~3 for cobalt and ~2 for nickel), which uses different His ligands for cobalt to bind to RcnR (vide infra).[125,127] Mutagenesis, lacZ assays, and XAS studies determined that nickel and cobalt are distinguished from noncognate metals by binding to RcnR using the N-terminal amine.[125,127] The nickel and cobalt complexes are further distinguished by the use of His3, which was found by XAS to be a ligand for cobalt, but not for nickel.[127]

In addition to Cys35, the N-terminus, and His3, RcnR has four other histidine residues (His33, His60, His64, and His67) that are potential metal ligands. Mutagenesis and lacZ assays determined that His3, His64, and Cys35 were necessary for nickel and cobalt responsiveness, and His60 was important only for cobalt responsiveness, indicating further differences in the use of His ligation by the two cognate metals.[125] RcnR forms three- or four-coordinate complexes with noncognate metals, copper(I) and zinc, involving three protein ligands and one anion from the buffer.[127]

Metal responsiveness in RcnR was altered through an H3E mutation, which resulted in a protein that was responsive to Zn(II) ions.[127] This mutation alters the zinc site from a four-coordinate site with an $(N/O)_2SBr$ ligand set in WT RcnR to an unusual six- or seven-coordinate (with a bidentate carboxylate) site with an $(N/O)_{5-6}S$ ligand donor atom set featuring a Zn−S bond distance of 2.61 Å, similar to the Ni−S distance in the WT protein. This result suggested that the H3E mutant protein is responsive to Zn(II) binding because the ion binds to the protein through the same points of attachment as the cognate metals and thus generates a protein conformation, or change in dynamics, that results in release of DNA.

The interactions that couple metal binding to the allosteric response seem to be the essential structural feature in CsoR/RcnR proteins. The copper(I)-responsive CsoR protein from *M. tuberculosis* binds Cu(I) with Cys36, His61, and Cys65,[171] which correspond to Cys35, His60, and His64, respectively, in RcnR. Nonetheless, InrS, a new member of the RcnR/CsoR family of proteins that has the CsoR ligand set with a cysteine residue in the position corresponding to His64 in RcnR, was shown to be nickel-responsive.[137] These results suggest that factors other than the first coordination sphere of the metal are responsible for metal-specific responses in the CsoR/RcnR family of metal-responsive transcriptional regulators.

NmtR and KmtR. Two nickel- and cobalt-responsive transcriptional regulators that belong to the ArsR/SmtB family have been identified in *M. tuberculosis*, NmtR and KmtR.[128,131] *M. tuberculosis* requires nickel for the enzyme urease and a hypothetical nickel-containing glyoxylase I enzyme.[8,130] Although NmtR has no amino acid sequence homology with RcnR and belongs to a distinct class of DNA-binding proteins, it resembles RcnR in several ways. Like RcnR, *M. tuberculosis* NmtR is responsive to both nickel and cobalt binding, controlling the expression of the P-type ATPase, NmtA, by regulating the *nmt* operon via release of DNA upon binding a cognate metal.[128,129] Also like RcnR, NmtR binds its cognate metals with higher coordination numbers and noncognate metals with lower coordination numbers. NmtR binds nickel and cobalt in six-coordinate and five- or six-coordinate geometries, respectively, and binds the noncognate metal ion zinc in a four-coordinate geometry.[129] In contrast to RcnR, no Cys ligation is involved in metal coordination, and mutagenesis and metal binding studies identified His3 as an important nickel ligand.[129] The H3Q-NmtR mutant protein showed more sensitivity to noncognate zinc regulation than to the cognate metals cobalt and nickel.[130] However, unlike RcnR, H3Q-NmtR could regulate protein−DNA interactions in response to nickel binding as well as the WT NmtR protein.[130] A recent NMR structure of apo-NmtR reveals that the apoprotein is in an "open conformation" and adopts a typical winged-helix fold common to the ArsR repressor family.[172] Ratiometric pulse chase studies support the binding of the N-terminal amine to nickel because the rate of amidation of the α-amino group of Gly2 is strongly reduced when nickel is bound compared with the apoprotein and zinc-bound protein.[172] Molecular dynamics simulations, quantum chemical calculations, and mutagenesis studies support a model for the binding of nickel to NmtR that features a six-coordinate geometry involving the N-terminal amine and the side chains of His3, Asp91, His93, His104, and His107.[130,172] Thus, there are two examples of metalloregulators that form six-coordinate sites with nickel that involve coordination of the N-terminal amine, which is emerging as key structural determinant in distinguishing nickel ions from other metals.

Nur. *Streptomyces coelicolor* Nur is a nickel-responsive member of the Fur family of transcriptional regulators that controls nickel homeostasis and oxidative stress response.[132] Nur negatively regulates the *sodF* gene that encodes FeSOD and the *NikABCDE* gene cluster encoding components of the nickel transporter.[132] Nur binds to the promoter regions of these genes in the presence of Ni(II) but not with Fe(II), Zn(II), Co(II), Cu(II), Mn(II), or Cd(II).[132] Nur also plays an

indirect role in the expression of the *sodN* gene, which encodes a NiSOD. Members of the Fur family are homodimeric proteins, with each monomer containing an N-terminal DNA-binding domain, a C-terminal dimerization domain, and a hinge region connecting the two domains.[133] The Nur crystal structure (Figure 7) reveals that there are two metal binding sites in each monomer: the M site and the nickel site. The M site, located at the domain interface, can coordinate either nickel or zinc.[133] There is some ambiguity regarding the structure of the nickel site, as the crystal structure showed that the nickel site is octahedral with three histidine ligands, His70, His72, and His61, and three oxygen atoms from malonate and ethylene glycol. Attempts to grow Nur crystals without malonate and dehydrating the crystals confirmed the presence of the three histidine ligands, but they could not identify the electron density for any water molecules.[133] Thus, the structure of the nickel site could be due in part to an artifact of the crystallization conditions.

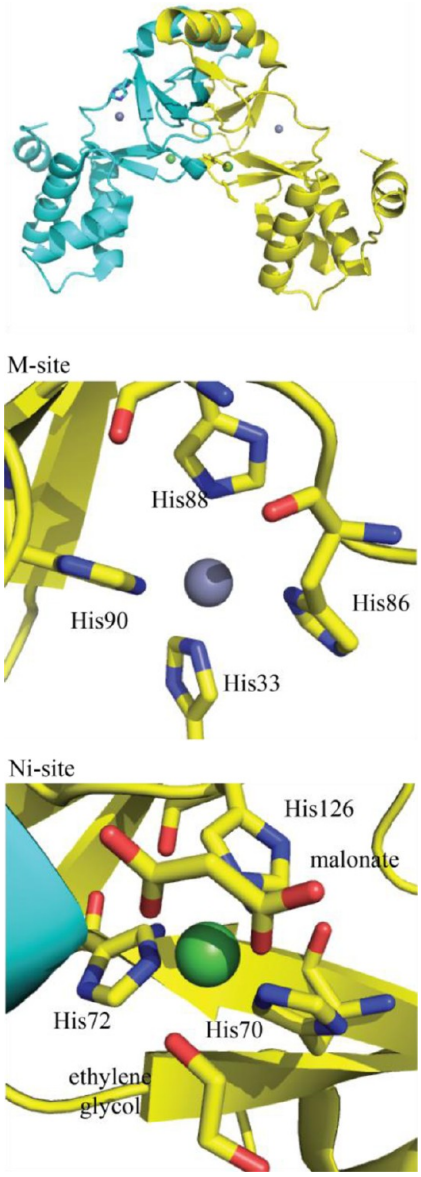

Figure 7. *S. coelicolor* Nur crystal structure (top) highlighting the M site with zinc bound (slate) and the nickel site with nickel bound (green) (PDB entry 3EYY). M site (middle) with zinc (slate) coordinated by His33, His86, His88, and His90. Nickel site (bottom) with nickel (green) coordinated by His70, His72, His126, ethylene glycol, and malonate.

CONCLUSION

Several themes regarding nickel recognition in trafficking proteins emerge from these studies. The ABC-type nickel importer, NikA, appears to recognize neither Ni(II) ions nor $Ni(H_2O)_6^{2+}$ ions, but rather a $Ni(L\text{-}His)_2$ complex that is recognized by the formation of particular H-bonds, which thus resembles recognition of specific peptides in peptide transporters.

Many of the proteins that do bind Ni(II) ions couple allosteric changes to the coordination number and/or ligand set adopted by the nickel complex, the details of which are just beginning to emerge. In a sense, both NikR and RcnR are examples of proteins that utilize extra ligands to create alternative metal-binding sites that overlap the cognate metal binding site but prevent noncognate metals from binding in the same fashion as cognate metals. There are also features that appear to distinguish proteins that repress transcription from those that derepress transcription in response to metal binding. Proteins that repress transcription in response to nickel binding (e.g., NikR and Nur) appear to possess two Ni(II) binding sites: one site with square planar geometry that is unique to Ni(II) coordination chemistry among the biologically significant transition metals and another site with octahedral geometry. In contrast, proteins in which Ni(II) binding leads to derepression (e.g., RcnR and NmtR) possess one nickel binding site, often recognize other similar metal ions [e.g., Co(II)], and utilize a six-coordinate nickel complex that involves coordination of the N-terminal amine to destabilize the protein−DNA interaction. Additional comparisons with other metalloregulators, such as those among RcnR, CsoR, and InrS, reveal that the nature of the metal complex formed may be relatively unimportant; rather, it is the mechanism that couples the formation of the complex to protein allostery that is the critical feature in the design of nickel metalloregulators.

Protein allostery also plays a role in trafficking proteins other than metalloregulators. Such proteins include the metallochaperone, HypA, where although there is little evidence that the nickel site structure is sensitive to pH, a structural zinc site undergoes ligand substitution in response to binding nickel and changes in pH. The protein structural change that presumably accompanies the change in the zinc site may be critical in directing nickel traffic to apo-urease under acid stress conditions, a key response required for the acid viability of *H. pylori*. The use of zinc in regulating proteins involved in metallocenter assembly, such as HypA,[39,42,45] HypB,[51,52] UreE,[89] and UreG,[173] is another emerging theme.

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