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Nickel-responsive transcriptional regulators†

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Nickel is an essential micronutrient for a large number of living organisms, but it is also a toxic metal ion when it accumulates beyond the sustainable level as it may result if and when its cellular trafficking is not properly governed. Therefore, the homeostasis and metabolism of nickel is tightly regulated through metal-specific protein networks that respond to the available Ni(II) concentration. These are directed by specific nickel sensors, able to couple Ni(II) binding to a change in their DNA binding affinity and/or specificity, thus translating the cellular level of Ni(II) into a modification of the expression of the proteins devoted to modulating nickel uptake, efflux and cellular utilization. This review describes the Ni(II)-dependent transcriptional regulators discovered so far, focusing on their structural features, metal coordination modes and metal binding thermodynamics. Understanding these properties is essential to comprehend how these sensors correlate nickel availability to metal coordination and functional responses. A broad and comparative study, described here, reveals some general traits that characterize the binding stoichiometry and Ni(II) affinity of these metallo-sensors.

Introduction

Some transition metal ions are essential micronutrients for living organisms. Their limited environmental availability and intrinsic toxicity represent important factors of evolutionary pressure to develop mechanisms for specific and selective metal ion accumulation and utilization. Indeed, metal homeostasis networks have been developed and ensure that appropriate concentrations of metal ions are maintained in the cytoplasm under physiological conditions. Concomitantly, tightly regulated intracellular metal trafficking mechanisms modulate the presence of the correct metal ion in metallo-proteins and sub-cellular compartments.[1] In particular, iron, copper, zinc, manganese, cobalt and nickel all have unique chemical and physical properties,

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so that, in general, metallo-proteins use a specific metal ion to carry out their function. The metal-specificity of cellular responses indicates that metal-binding proteins are able to choose the correct cofactor for a specific biological function among all available metal ions. In some cases, the metabolisms of different metal ions can overlap, implying that the homeostasis of a single ion should not be considered independently of the overall intracellular metal ion content.[2] The introduction of the term *metallome* thus describes the full content, distribution and concentration of metal ions found in a certain organism.[3] The study of the metallome would eventually lead to the understanding of how life evolved and how cellular metabolisms adapted to the presence of diverse micronutrients.

A correct balance of metal uptake, trafficking and extrusion is also essential to establish relationships between prokaryotic and eukaryotic organisms, such as symbiosis and pathogenesis.[4] Similarly, competition for metal ions is imperative for host–pathogen interactions. Mammalian hosts often use metal withholding by means of metal binding proteins to prevent pathogen growth by decreasing metal ion concentration to levels incompatible with bacterial life.[5] To compensate, bacteria synthesize proteins and/or smaller molecules to chelate metal ions and to subtract them from the host systems, a notable example being represented by the systems to acquire iron.[6] In addition, bacteria sense the presence of low environmental levels of metal ions and respond by inducing the expression of a wide variety of toxins and other virulence factors.

The crucial players of metal-based metabolic networks are specific metal-responsive transcriptional regulators, generally defined as metalloregulatory proteins or *metal sensors*, which couple specific metal ion binding with a change in their DNA binding affinity and/or specificity, thus translating the concentration of a certain metal ion into a change in genetic expression.[7] In general, specific interactions between proteins and metal ions, driven by the coordination chemistry and geometry of metal-binding sites, are propagated away from the specific metal binding site through changes in protein structure and/or dynamics along the protein backbone, resulting in a change in the DNA binding affinity of the protein. This event causes repression, derepression, induction or enhancement of transcription of genes that codify metal ion efflux or uptake pumps, membrane permeases, soluble metallo-chaperones, metal-accumulation proteins, metal-dependent enzymes, and metal-dependent transcriptional regulators.[8] This process, in turn, induces a finely tuned metabolic response driven by metal ions, which include the coordinated control of the entire machinery of metallo-enzyme synthesis and activation, as well as the systems for homeostasis that involve competitive metal ion uptake, intracellular accumulation and, ultimately, extrusion. In addition, some metal sensors also control metabolic processes other than metal homeostasis, such as oxidative stress resistance, toxin synthesis, and acid adaptation.[1b,9]

In the present review, we focus on how specific proteins bind Ni(II) and consequently influence their interaction with DNA, and how this event can impact the fate of genetic expression, leading to specific transcriptional outputs. Nickel is found in eubacteria, archaebacteria, fungi, and plants, where nickel-containing

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Stefano Ciurli received a laurea degree in Chemistry from the University of Pisa (Italy) in 1986, with Prof. Floriani, and a PhD degree in Chemistry from Harvard University (MA, USA) in 1990, with Prof. Holm. After two years of postdoctoral studies with Prof. Bertini and Prof. Luchinat, he was appointed Associate Professor and then Full Professor of General and Inorganic Chemistry in 2001 at the University of Bologna (Italy). His research is focused on the structure–function relationship of metal-based biocatalysis, metal ion cellular trafficking, and metal-mediated protein–DNA interactions involved in metal ion sensing and metal-dependent gene regulation.

enzymes catalyse a wide assortment of redox and non-redox reactions.[10] In particular, nine nickel-dependent enzymes are currently known: urease,[10c] methyl coenzyme-M reductase,[11] carbon monoxide dehydrogenase and acetyl coenzyme-A synthase,[12] hydrogenase,[13] superoxide dismutase,[14] glyoxalase I,[10c] acireductone dioxygenase,[10c] and lactate racemase.[15] In a limited number of eukaryotic systems, nickel ions are required for the urease enzyme, whereas the rest of the nickel-containing enzymes determined so far are found in prokaryotes. Nickel ions are also potentially toxic and can damage the cells in several ways.[16] In particular, four mechanisms of nickel toxicity have been proposed: nickel could (i) replace the essential metal of metalloproteins, (ii) bind to catalytic residues of non-metalloenzymes, (iii) bind outside the catalytic site of an enzyme to inhibit it allosterically, and (iv) indirectly cause oxidative stress.[16d] Therefore the distribution and accumulation processes of nickel must be tightly controlled. Thus, in addition to the enzymes that employ Ni(II) as chemical prosthetic groups, nickel depending organisms also express a variety of proteins that contribute to Ni(II) homeostasis by assembling metallo-centres, importing or exporting the metal, transporting nickel ions within the cell, or regulating the expression of the factors involved. The features of representative nickel-responsive transcriptional regulators, as well as the molecular basis of nickel–protein and protein–DNA interactions, are discussed in the present review on the basis of the available structural information. An overview of the recent advances in the understanding of how these proteins specifically choose nickel ions among the intracellular metal ion pool, as well as how they allosterically respond to their effector binding, is given.

NikR

NikR is a transcription factor that regulates the expression of gene coding for proteins involved in nickel metabolism. It is a highly homologous protein, found in *ca.* 30 species of bacteria and archea. The Ni(II)-bound NikR from *Escherichia coli* (*Ec*NikR) binds to DNA and represses the transcription of the *nikABCDE* operon, which codes for a specific Ni(II) membrane uptake ABC transporter.[17] On the other hand, NikR from *Helicobacter pylori* (*Hp*NikR) is a pleiotropic regulator of several genes,[18] acting as a nickel-dependent repressor of its own gene and of the gene coding for the Ni(II) permease NixA, responsible for the import of Ni(II), as well as a nickel-dependent activator of genes coding for the pathogenic factor Ni(II)-containing urease.[19] In *H. pylori*, the disruption of the Ni(II)–chaperone network, involved in metal ion delivery to the enzyme, results in a deregulation of the Ni(II)-dependent transcriptional regulation by NikR,[20] suggesting that intracellular metal ion availability influences the activity of this metal sensor, in a mechanism that could be operative in other cases as well.

Several crystal structures of NikR[21] have established that this protein is a homo-tetramer, made of a dimer of dimers, constituted by two domains (Fig. 1A). One domain is the central metal-binding domain (MBD), made of the C-terminal portion of the protein responsible for tetramerization. This domain hosts four regulatory metal binding sites symmetrically located at the tetramerization interface, where diamagnetic Ni(II) ions bind three fully conserved histidines and one cysteine residues in a square planar coordination geometry (Fig. 1B). The MBD is flanked by two peripheral DNA-binding domains (DBDs), separated by flexible linkers. Each DBD is made by the dimerization of the N-terminal portion of the protein and features a ribbon-helix–helix motif for DNA binding typical of prokaryotic transcription factors[22] in which two anti-parallel N-terminal β-strands from opposite protomers make a two-stranded anti-parallel β-sheet that contacts the major groove (Fig. 1A).

Three distinct conformations of NikR have been observed in the solid-state, characterized by the position of the DBDs with respect to the central MBD, depending on the conformation of the flexible link between the domains: *open*, *trans*, and *cis* (Fig. 1A). The crystal structure of the *Ec*NikR–DNA complex has shown that the *cis* conformation of the Ni(II)-bound protein is able to bind DNA with the MBD that keeps the DBDs at the right distance to contact one-half-site of a two-fold symmetric DNA operator (Fig. 1A).[21c]

The metal binding activity of NikR and its effect on the reactivity of the protein toward DNA binding has been the object of several investigations. Initially, the dissociation constants for Ni(II) binding to MBD were estimated through competition experiments in the presence of the chelating competitor EGTA, which gave dissociation constants in the pM range.[24] These values are possibly affected by protein interactions with the competitor molecule, and depend strictly on the data chosen for the competitor-Ni(II) binding constant among the several and largely different values available in the literature.[25] Subsequently, a calorimetry-based titration method, which does not require additional metal chelators present in solution beside the protein itself, was applied for *Hp*NikR.[26] These experiments indicated that the four Ni(II) binding sites in the tetramer are distinct, and bind Ni(II) in a 2 + 2 stoichiometry, with dissociation constants of 12 and 125 nM at pH 7.[26] The pH dependence of the binding constants in the pH range 6.5–8.0 revealed an increase up to two orders of magnitude, attributed to the deprotonation event of the Ni(II)-binding cysteine.[26] Similar dissociation constants have been reported for *Ec*NikR using different techniques.[27] A titration of Ni(II) onto *Ec*NikR using ITC, under conditions identical to those used for *Hp*NikR,[26] also indicated a 2 + 2 binding mode, with dissociation constants of 25 nM and 400 nM (Fig. 1B and Table 1-SI, ESI†). This result suggests a general conservation of stoichiometry, affinity and interaction mode of Ni(II) with NikR proteins from different sources.

The effect of Ni(II) binding to the MBD of *Hp*NikR propagates away from the metal sites, resulting in an increase of the protein affinity to DNA and reaching a dissociation constant of 56 nM, as determined by calorimetric titrations.[28] This effect, possibly occurring through a hydrogen-bonding network, was consistently observed as endothermic peaks in the ITC titration curve of both *Hp*NikR[26] and *Ec*NikR (see Fig. 1-SI, ESI†), corresponding to the quenching of the intrinsic fluorescence of the tryptophan residue located in the inter-domain linker in the presence of Ni(II).[28] This event, specific for Ni(II), couples the metal-specific stereo-electronic preferences of Ni(II) with

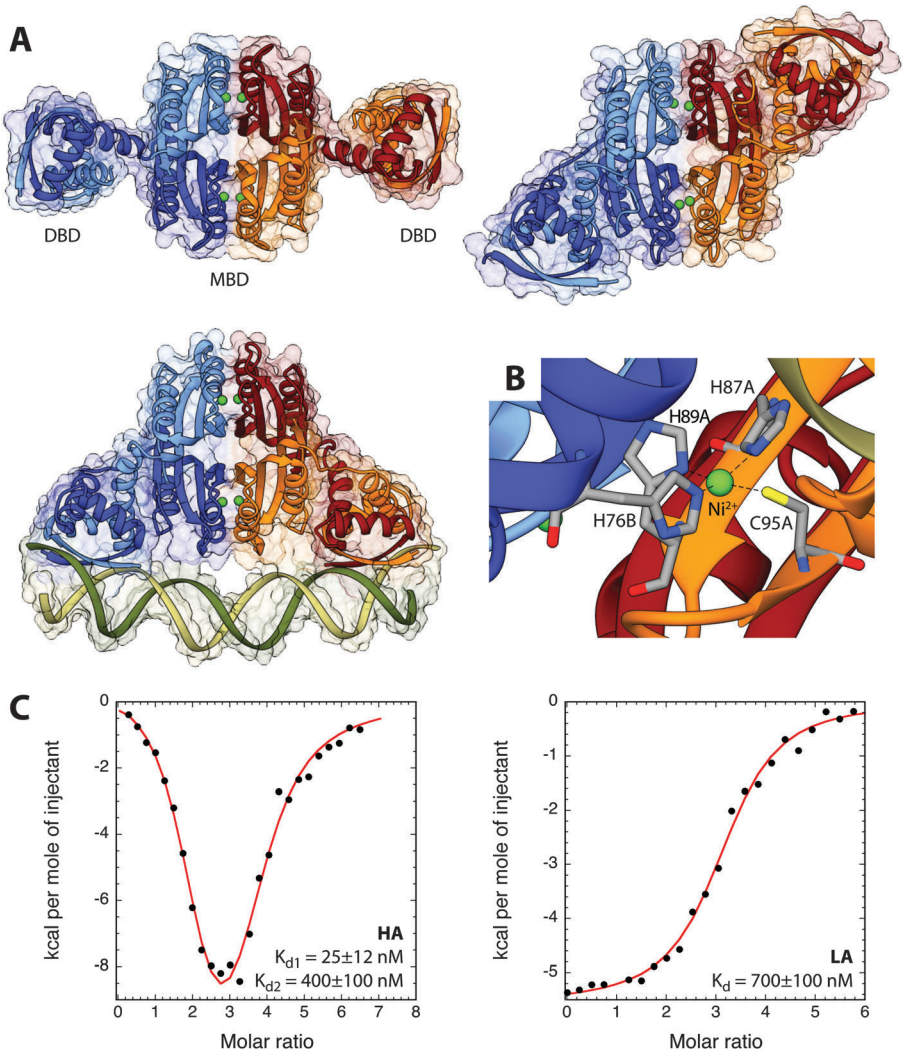


Fig. 1 (A) Ribbon scheme and solvent-excluded surface of NikR in the *open*, *trans* and *cis* conformation (PDB codes: 2HZA, 2BJ8, and 2HZV, respectively). The monomers are coloured in blue, light blue, red, and orange, while Ni(II) ions are reported as green spheres. DNA is depicted in light and dark green. (B) Details of the Ni(II) binding site. The molecular graphics images in this and all subsequent figures were produced using the UCSF Chimera package.[23] (C) ITC titration data for the binding of Ni(II) to *Ec*NikR at 25 °C in 20 mM Hepes buffer pH 8.0, containing 150 mM NaCl. In the left panel, normalized heats of reaction for the higher affinity (HA) Ni(II) binding events (2 + 2) were obtained by integrating the raw data generated by 10 μL injections of a $NiSO_4$ solution (500 μM) into a protein solution (1.5 μM). In the right panel, normalized heats of reaction for the four lower affinity (LA) Ni(II) binding events were obtained by integrating the raw data generated by 10 μL injections of $NiSO_4$ solution (300 μM) into a protein solution (1 μM) pre-incubated with 4 μM Ni(II). The continuous lines represent the best fit of the integrated data, obtained by a nonlinear least-squares procedure. The details of the measurements are provided as ESI.† The dissociation constants are indicated here and in Table 1-SI (ESI†).

the structural and/or dynamic properties of the protein, rendering it able to bind DNA. On the other hand, the non-cognate metal ions Zn(II) and Co(II) bind with different affinities and stoichiometries and adopt a variety of alternate geometries and ligands that prevent the correct response to occur.[21e,28]

The way through which Ni(II) binding propagates the information for DNA binding at the MBD along the protein chain to the DBDs has been investigated both experimentally, using small angle X-ray scattering (SAXS)[29] and NMR[30] experiments, and computationally, using atomistic molecular dynamics simulations.[30,31] The conclusions demonstrate the occurrence of an ensemble of interconverting structures in solution, spanning among the *open*, *trans* and *cis* conformers, both for the apo and the holo-protein. This observation suggests that Ni(II) binding does not induce a conformational rearrangement of the protein towards a specific *cis* conformation able to bind DNA, but rather unlocks the movement of the two peripheral N-terminal DNA-binding domains with respect to the central C-terminal metal binding domain.[30,31b] Consistently with this hypothesis, the crystallographic analysis of holo-*Hp*NikR indicated that, while the MBD is well ordered in holo-*Hp*NikR, the position of the DBDs is so disordered that they do not appear in the crystal structure.[21g] The Ni(II)-induced increased mobility of the DBDs with respect to the MBD would allow the protein to more frequently sample the *cis* conformation and specifically read the DNA sequence, locking the protein in the *cis* conformation

only in the presence of the correct DNA sequence, through an induced fit mechanism.[30,32]

Additional binding of Ni(II) to lower affinity sites, observed both for *Ec*NikR (Fig. 1B and Table 1-SI, ESI†) and *Hp*NikR,[28] appears to improve the DNA binding affinity of *Ec*NikR,[17b,24b,33] while it has a marginal effect on DNA binding to *Hp*NikR and only on lower affinity promoters.[28,34]

RcnR

In addition to NikR, *E. coli* produces a second Ni(II)-dependent regulator, named RcnR. In particular, while NikR controls the expression of Ni(II) imported through the NikABCDE transporter, RcnR is a Ni(II) and Co(II) sensor that regulates the expression of nickel and cobalt efflux proteins,[35] namely RcnA, a membrane permease,[36] and RcnB, the associated periplasmic protein.[37] An important connection between this system and iron homeostasis has also been reported, with the regulation of the *rcnA* gene expression deriving from the interplay of nickel, cobalt and iron homeostasis through the ferric uptake regulator (Fur).[38]

RcnR belongs to the CsoR/RcnR family of transcriptional repressors,[7a] which also includes the Ni(II) sensors DmeR (a divalent metal efflux repressor)[39] and InrS (internal nickel-responsive repressor).[40] RcnR shares less than 20% sequence similarity with CsoR (Fig. 2A). Many of the CsoR/RcnR proteins control metal ion efflux through specific membrane transporters, exerting transcriptional derepression of their genes in the presence of the cognate metal ion. No structure of RcnR is available, but the protein has been proposed to show an all α-helical fold similar to that of CsoR.[41]

A computational model of *Ec*RcnR, obtained using an established protocol (see ESI,† for details),[30,42] suggests the presence of three α-helices in the monomer: α1 (residues 5–31), α2 (36–71), and α3 (74–89) (Fig. 2B). Each monomer is in contact with a second protein chain *via* the three α-helices to form a dimer. The tetrameric oligomer is obtained by the interaction between the two helices α1 from one dimer with the corresponding helices from the second dimer. The similarity with CsoR also suggests that the protein acts as a tetramer for DNA binding. The protein has been indeed shown to be mainly a tetramer in solution from ultracentrifugation analysis.[35b] Light scattering experiments indicated an average molar mass of 33 ± 2 kDa for the apo-protein at 5 μM concentration (theoretical molar mass is 10.1 kDa for the monomer), suggesting that the protein undergoes an oligomerization equilibrium in solution (see Fig. 2-SI, ESI†). Similarly, RcnR showed a concentration-dependent stability in chemical denaturation experiments, indicating that oligomer dissociation might depend on protein concentration, and that RcnR likely couples dissociation and unfolding.[35b]

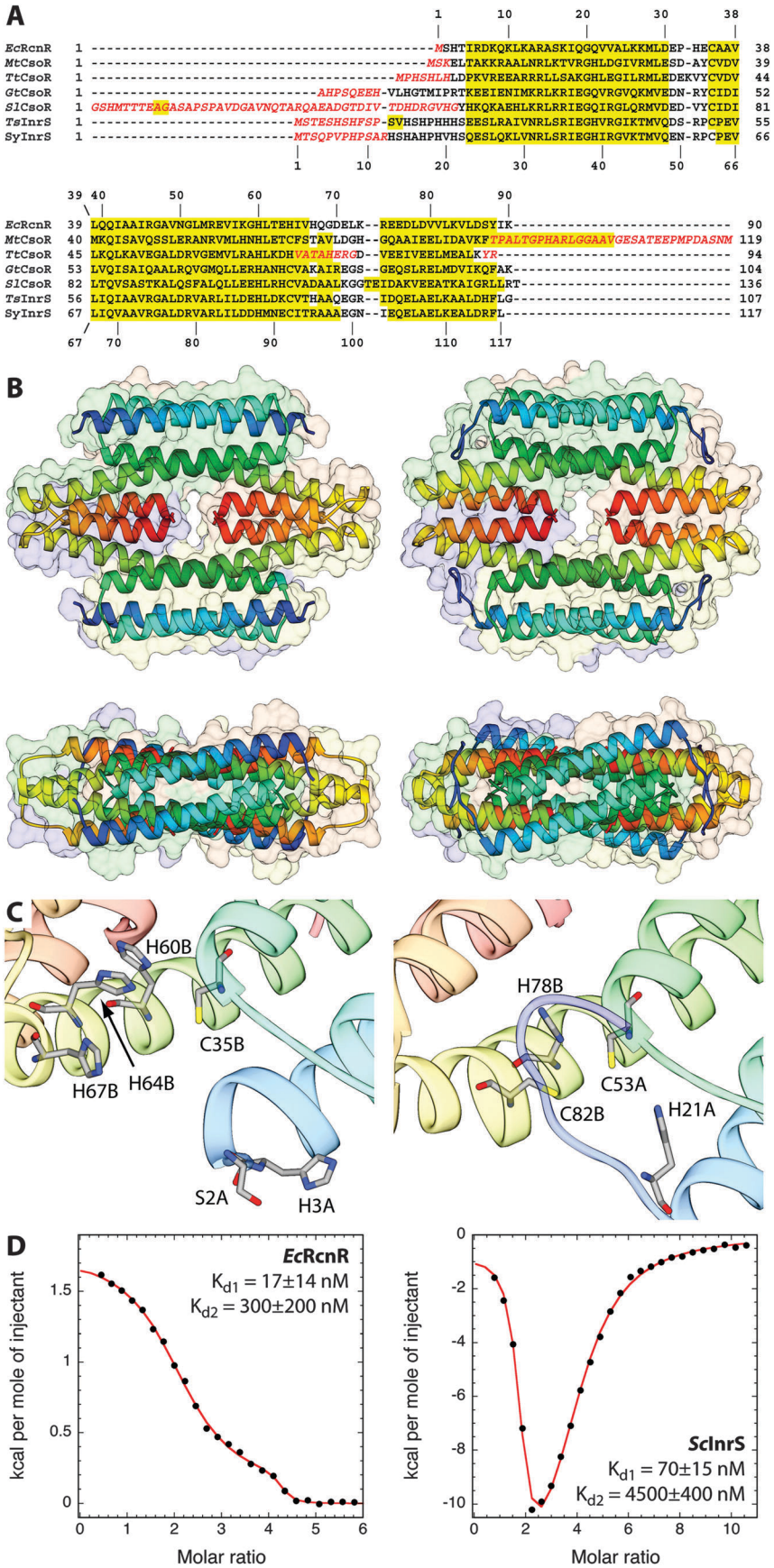
*Ec*RcnR binds several metal ions *in vitro*, but only Ni(II) and Co(II) de-repress the transcription of RcnA, acting as cognate metal ions.[35b] Metal binding appears to cause a conformational modification that decreases the protein affinity to DNA, thus inducing a disruption of the protein–DNA complex and resulting in the expression of RcnAB. Based on X-ray absorption spectroscopy (XAS), it has been shown that *Ec*RcnR forms six-coordinate pseudo-octahedral complexes with the cognate metal ions Ni(II) and Co(II),[35b] and four-coordinate complexes with non-cognate Zn(II) and Cu(I).[44] Both cognate metals adopt a $(N/O)_5S$ coordination shell that includes the single Cys35 residue.[35b,44] Mutagenesis studies, physiological assays and electronic absorption spectroscopy also suggested that the Ni(II) and Co(II) sites include the N-terminus amino group, while this ligand is not involved in the binding site of Zn(II) and Cu(I).[35b,44] The side chain of His3 appears to distinguish between Ni(II) and Co(II), acting as a ligand for the latter but not for the former.[44] The imidazole rings of His60 and His64 have been suggested to be additional ligands for Co(II),[35b,45] with His67 playing a possible indirect role in the binding site stabilization, while His64 appears to be important for Ni(II) binding.[45]

Metal binding titration in the presence of a fluorescent metal competitor indicated that RcnR binds Ni(II) with a dissociation constant of 25 nM using a model with four non-interacting sites.[35b] Calorimetric titrations performed in our laboratory using a similar experimental protocol used for *Hp*NikR (see ESI,† Fig. 2C and Table 1-SI, ESI†) confirmed this range of affinity for Ni(II) binding to RcnR. In addition, this metal ion binds the protein tetramer with a 2 + 2 stoichiometry, similarly to NikR, with measured dissociation constants of 17 nM and 300 nM for the two binding events. Interestingly, Ni(II) binding to RcnR produces a heat effect that is observed only when the protein is at a relatively high concentration (40 μM tetramer), while it is negligible when the protein is at lower concentrations (7–15 μM tetramer). This concentration-dependent metal binding suggests that Ni(II) preferentially binds to the tetrameric form of the protein. Accordingly, light scattering experiments, performed in the presence of Ni(II) under the same experimental conditions and concentrations used for the apo-protein, showed a calculated molar mass of 45 ± 1 kDa, in full agreement with the theoretical molar mass of the tetramer (see Fig. 2-SI, ESI†). This supports a Ni(II)-driven stabilization of the dimer of dimers, and is consistent with the higher stability observed for the protein in the presence of metal ions.[35b]

The binding affinities thus measured for these Ni(II) sensors in *E. coli*, which are devoted to detecting too little (NikR) or too much (RcnR) Ni(II), indicate that the physiological cytoplasmic concentration of Ni(II) is in the 10–500 nM range for this organism. We speculate that similar levels could be observed in other organisms as well.

InrS

InrS is another Ni(II)-regulated sensor belonging to the CsoR/RcnR family (Fig. 2A and B). This protein from *Synechocystis* PCC6803 (*Sy*) regulates the production of NrsD, an efflux protein in the inner membrane.[40] *Sy*InrS migrates as a tetramer in gel filtration experiments, and, similarly to *Ec*RcnR, binds four Ni(II) or Co(II) ions per tetramer.[40] Competition experiments, using the Ni(II) chelating ligands NTA, EGTA and EDTA, estimated a dissociation constant of *Sy*InrS for Ni(II) in the sub-picomolar range (2×10^{-14} M).[40] On the other hand,



data obtained on the same protein using calorimetric experiments, in the absence of possible artefacts due to the presence of the exogenous competitors and under buffer conditions similar to those previously used in the case of *Hp*NikR, *Ec*NikR, and *Ec*RcnR (see above), revealed dissociation constants in the nanomolar/micromolar range (Fig. 2C and Table 1-SI, ESI†), by analogy with the observations reported for the homologue RcnR. Moreover, similarly to NikR and RcnR, a 2 + 2 binding mode for *Sy*InrS was also observed, with the two binding events featuring dissociation constants of 70 nM and 4.5 μM, respectively.

The electronic absorption spectral properties of Ni(II)–*Sy*InrS are different from those of *Ec*RcnR and are suggestive of Ni(II) coordinated in a square planar geometry, analogous to NikR but involving two Cys and two His residues.[40] The sequence alignment of *Sy*InrS and *Ec*RcnR indicates the conservation of His21, Cys53, and His78 (*Sy*InrS numbering), corresponding to His3, Cys35 and His60 in *Ec*RcnR, while Cys82 in *Sy*InrS replaces His64 found in *Ec*RcnR (Fig. 2A). Site-directed mutagenesis studies have indeed identified Cys53, Cys82 and His78 as Ni(II) ligands, while it remains unclear whether or not His21 (corresponding to His3, a residue not involved in Ni(II) binding in *Ec*RcnR) is a ligand for Ni(II).[46] Five additional His residues are found in the N-terminal region of *Sy*InrS as compared to *Ec*RcnR, and these could also play a role in metal sensing and/or storage.

NmtR and KmtR

The metal sensors NmtR and KmtR belong to the ArsR/SmtB family of transcriptional regulators[47] and are responsible for Ni(II) homeostasis in *Mycobacterium tuberculosis* (*Mt*), a ureolytic pathogen that requires nickel for the activity of urease, a virulence factor. By analogy with RcnR, these proteins function as repressors in the *apo*-form, recognizing and binding DNA within the promoter region, and inhibiting the concomitant interaction and activity of RNA polymerase. Ni(II), Co(II), and Zn(II) function as inducers, driving *Mt*NmtR dissociation from DNA and de-repressing the transcription of genes coding for the P-type ATPase metal transporter NmtA and for a cation diffusion facilitator (CDF).[47] The ability of metals to allosterically regulate specific DNA binding by NmtR follows the Ni(II) > Co(II) > Zn(II) trend both *in vivo*[47a] and *in vitro*.[48]

All studied members of the ArsR/SmtB family are stable dimers and share a general fold comprising at least five α-helices and a two- or three-stranded β-sheet. Helices α3 and α form two winged helix-turn-helix motifs per dimer, responsible for DNA recognition and binding. The other three helices are involved in hydrophobic interactions within the monomer responsible to build and orient the DNA binding motif. In addition, helices α and α5 are involved in dimerization. Despite their generally similar folds found within the different members of the family, the position and coordination of metal binding sites are very different in different ArsR/SmtB proteins.[7b,9a] The dimeric state of *Mt*NmtR has been confirmed by analytical equilibrium sedimentation ultracentrifugation experiments.[48]

XAS data indicate that *Mt*NmtR binds Ni(II) in a pseudo-octahedral six-coordinate geometry that includes three histidine imidazole N atoms, one of which being His3, while Zn(II) is found in a four-coordinate-geometry, and Co(II) appears to bind five or six ligands, based on UV/visible spectroscopy.[48] In contrast to *Ec*RcnR, no Cys residues were found in the coordination shell of these ions.[48]

The NMR structure of apo-*Mt*NmtR reveals the typical core winged-helix fold of the ArsR family, with distinct long flexible N- and C-terminal tails, in an open conformation[49] (Fig. 3). On the basis of the protein solution structure and other biophysical methods, as well as molecular dynamic simulations, NmtR was proposed to bind Ni(II) in two identical sites that comprise Asp91 and His93 from one chain, and His3, His104, and His107 from the other chain, the latter also involved in providing the N-terminal $-NH_2$ group as an additional ligand.[49] Because of the length and mobility of the N-terminal tail, it should not be excluded, however, that the N-terminal residues found in the same chain of Asp91 and His93 are instead recruited for Ni(II) binding. NMR analysis revealed that, in the presence of Ni(II), some regions at the protein–DNA interface acquire higher mobility, suggesting that a Ni(II)-driven increase of protein dynamics is responsible for protein dissociation from DNA.[49] Therefore, as in the case of NikR, a metal-driven change in protein flexibility, and not a metal-induced specific conformational change, appears to be responsible for the functional modification of the transcription factor affinity to DNA, suggesting that this might be a general strategy used for other metal-sensing systems as well.

Fig. 2 (A) Alignment of the sequences of *Ec*RcnR, InrS from *Thermosynechococcus* sp. NK55a (*Ts*InrS) and *Synechocystis* PCC6803 (*Sy*InrS), as well as CsoR from *Mycobacterium tuberculosis* (*Mt*CsoR), *Thermus thermophiles* HB8 (*Tt*CsoR), *Geobacillus thermodenitrificans* NG80-2 (*Gt*CsoR), and *Streptomyces lividans* (*Sl*CsoR). The secondary structure indication (α-helix, yellow) was derived from PROMALS3D[43] for *Ec*RcnR, *Ts*InrS and *Sy*InrS, and from the crystal structures of CsoR (PDB codes: 2HH7, 3AAI, 4M1P, and 4ADZ for *Mt*CsoR, *Tt*CosR, *Gt*CsoR, and *Sl*CsoR, respectively). Residues at the N- and C-termini, not solved in the crystal structures and shown in red, were not modelled. (B) Ribbon schemes and solvent-excluded surfaces of tetrameric *Ec*RcnR (left) and *Sy*InrS (right) model structures. The models were calculated using the sequence alignment reported in panel (A) and a standard modelling procedure previously used.[30,42] The details of the model calculation are provided as ESI.† The ribbons of each monomer are coloured from blue in the proximity of the N-terminal to red at the C-terminus, while the surfaces are coloured to distinguish the four monomers. The orientation in the bottom panels is obtained by a rotation of 90° around the major horizontal axis of the orientation in the top panels. (C) Details of the proposed Ni(II) binding region in the *Ec*RcnR (left panel) and *Sy*InrS (right panel) model structures. (D) ITC titration data for the binding of Ni(II) to *Ec*RcnR (left panel) and *Sy*InrS (right panel) at 25 °C in 20 mM Hepes buffer (pH 7.0, 1 mM TCEP, and 150 mM NaCl for *Ec*RcnR; pH 7.8, 5 mM TCEP, and 300 mM NaCl for *Sy*InrS). Normalized heats of reaction for Ni(II) binding events (2 + 2) were obtained by integrating the raw data generated by injecting 10 μL of a $NiSO_4$ solution (1.2 mM for RcnR and 0.5 mM for InrS) into a protein solution (40 μM for RcnR and 10 μM for InrS). The continuous lines represent the best fit of the integrated data, obtained by a nonlinear least-squares procedure. The details of the measurements are provided as (ESI†). The dissociation constants are indicated here and in Table 1-SI (ESI†).

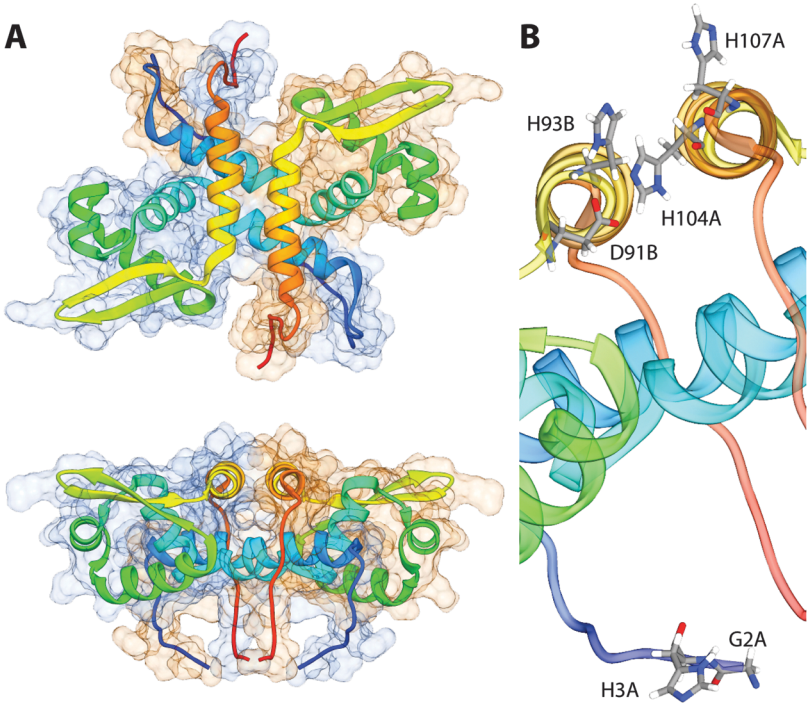


Fig. 3 (A) Ribbon scheme and solvent-excluded surface of the dimeric *Mt*NmtR (NMR structure, PDB code: 2LKP). The ribbons of each monomer are coloured from blue in the proximity of the N-terminal to red at the C-terminus, while the surfaces are coloured in orange and light blue to distinguish the two monomers. The orientation in the bottom panel is obtained by a rotation of 90° around the major horizontal axis of the orientation in the left panel. (B) Details of the proposed Ni(II) binding residues in *Mt*NmtR.

Different from NmtR, KmtR possesses a peculiar hybrid metal binding site, with residues from both α and α3 helices that contribute to building the ion coordination environment.[47b] The presence of two Ni(II)/Co(II) sensors of the ArsR/SmtB family in the proteome of *M. tuberculosis* has been rationalized on the basis of the different binding affinity of the two proteins for cognate metal ions, which extends the range of metal ion concentrations sensed by the bacterium.[47b] Indeed, KmtR has a tighter affinity for Ni(II) and Co(II) than NmtR,[47b] allowing these transcription factors to differentially regulate specific genetic patterns at different concentrations of the two ions: KmtR thus appears to detect low metal ion levels and release metal export *via* a CDF-family transporter, whereas NmtR allows transcription of the P1-type ATPase NmtA only when a higher threshold of these metals is reached.

Nur

The Ni(II)-sensor Nur belongs to the Fur family of metal-responsive transcriptional regulators.[50] This family is found in the genome of all Gram-negative bacteria with the only exception of the plant symbiont *Rhizobium* and some close organisms.[51] The first discovered member and the archetype of the family is the *E. coli* Fe(II)-dependent transcriptional repressor Fur.[52] Fur-like proteins generally function as holo-repressors and, in some cases also as apo-repressors, binding to DNA operators in the target core promoters.[53] Therefore, metal ions generally act as co-repressors of the gene transcription. Consistently, in the presence of Ni(II), but not in Fe(II), Zn(II), Co(II), Cu(II), Mn(II) or Cd(II), Nur from *Streptomyces coelicolor* (*Sc*Nur) represses the expression of *nikABCDE* operon encoding components of the ABC-type transporter as well as the *sodF* gene that encodes a Fe-containing SOD.[50a] *Sc*Nur also activates the expression of the *sodN* gene encoding a Ni-containing SOD, possibly in an indirect way.[50a]

All structurally characterized proteins of the Fur family share a general dimeric fold, with each monomer containing a N-terminal winged helix-turn-helix DNA binding domain (DBD) connected to a C-terminal dimerization domain through a short linker. Consistently, the same fold was observed in the crystal structure of *Sc*Nur (Fig. 4).[54] As other members of the Fur family, this protein contains two different metal binding sites, the M-site and the Ni-site, featuring a distinct specificity for Ni(II) as determined crystallographically. In the crystal structure, the M-site is found at the domain interface and involves His33, His86, His88, and His90 (Fig. 4B). In this site, the metal ion, modelled as Ni(II), is found in a square-planar geometry. This site appears to be able to also accommodate Zn(II), based on metal analysis and multiple anomalous dispersion crystallographic data. A similar site was also observed in *Pseudomonas aeruginosa* (*Pa*) Fur, with conserved metal binding residues.[50b,55] Differently, the Ni-site, only containing Ni(II) bound with an octahedral geometry at the domain interface, is unique for Nur. This Ni-site involves His70, His72, and His126 together with three oxygen atoms from a malonate anion and ethylene glycol (Fig. 4B), ligands recruited from the crystallization buffer and likely being water molecules *in vivo*. The Ni(II) coordination in the

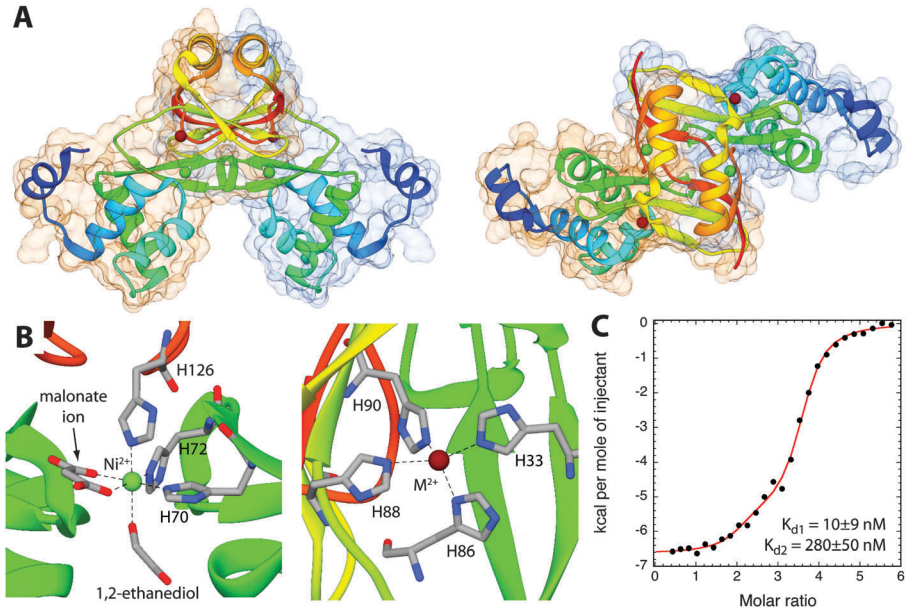


Fig. 4 (A) Ribbon scheme and solvent-excluded surface of dimeric *ScNur* structure (PDB code: 3EYY). The ribbons of each monomer are coloured from blue in the proximity of the N-terminal to red at the C-terminus, while the surfaces are coloured in orange and light blue to distinguish the two monomers. Ni(II) and Zn(II) ions are reported as green and dark red spheres, respectively. The orientation in the right panel is obtained by a rotation of 90° around the major horizontal axis of the orientation in the left panel. (B) Details of the Ni(II) (left) and Zn(II) (right) binding sites. (C) ITC titration data for the binding of Ni(II) to Nur at 25 °C in 20 mM Hepes buffer pH 8.0, containing 150 mM NaCl. Normalized heats of reaction for Ni(II) binding events were obtained by integrating the raw data generated by injecting 10 μL of a $NiSO_4$ solution (150 μM) into a protein solution (6.5 μM). The continuous lines represent the best fit of the integrated data, obtained by a nonlinear least-squares procedure. The details of the measurements are provided as (ESI†). The dissociation constants are indicated here and in Table 1-SI (ESI†).

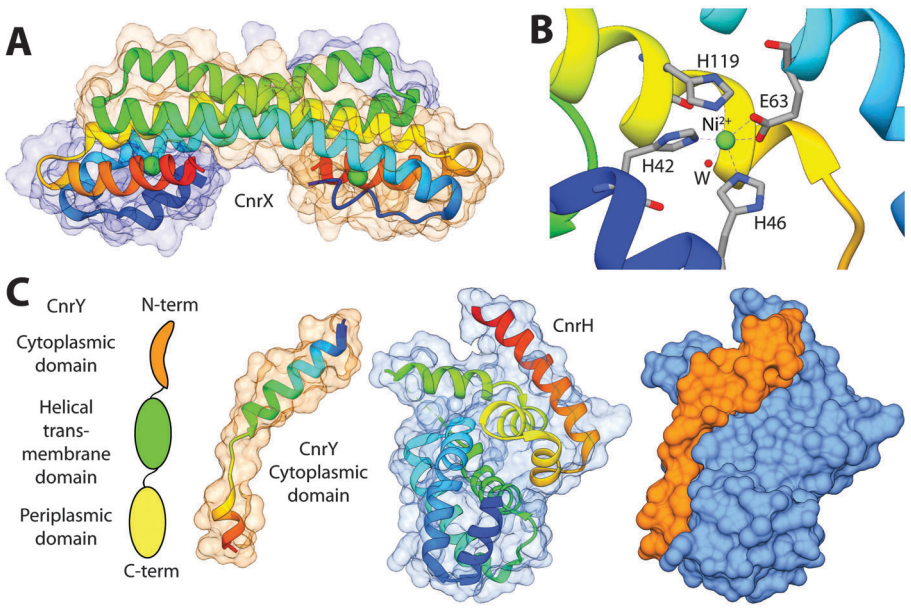


Fig. 5 (top) Ribbon scheme, solvent-excluded surface and details of the Ni(II) binding site of the dimeric *Cm*CnrX structure (PDB code: 3ZG1). The ribbons of each monomer are coloured from blue in the proximity of the N-terminal to red at the C-terminus, while the surfaces are coloured in orange and light blue to distinguish the two monomers. Ni(II) ions are reported as green spheres. (bottom) *Cm*CnrYH complex. *Cm*CnrY is composed by three domains (left panel) of which only the cytoplasmic domain has been crystalized in complex with the structure of *Cm*CnrH (PDB code: 4CXF). The central and right panels report the ribbon scheme and the solvent-excluded surface of CmCnrY and CmCnrH. The ribbons of each protein are coloured from blue in the proximity of the N-terminal to red at the C-terminus, while the surfaces are coloured in orange and light blue to distinguish the two proteins. The structure in the central panel has been obtained by translating the *Cm*CntY structure found in the crystal structure of the *Cm*CnrYH complex along the horizontal axis.

solvent-exposed Ni-site has been suggested to induce low binding affinity, even though its full occupancy by nickel appears to suggest the opposite.[54] Furthermore, the specificity of the Ni-site for Ni(II), coupled with the affinity of the M-site for zinc, strongly suggests that the Ni-site determines the nickel-responsive activation of Nur.[54]

Calorimetric titrations to determine the Ni(II) binding affinity and stoichiometry of *Sc*Nur, performed in our laboratory (see ESI†), are consistent with a 2 + 2 stoichiometry for the protein dimer, with the two binding events occurring with dissociation constants of 10 nM and 280 nM (Fig. 4 and Table 1-SI, ESI†). These data suggest a model that involves Ni(II) binding to the Ni-site and the M-site with higher and lower affinity, respectively.

The stoichiometry and the values of the dissociation constants determined for *Sc*Nur are once again consistent with all observations so far obtained for the other Ni(II) sensors, suggesting a common strategy for Ni(II) sensing by transcription regulators.

CnrYXH

The CnrYXH complex from *Cupriavidus metallidurans* (*Cm*) is an example of a three-component signal transduction complex, required for sensing the periplasmic Ni(II) and Co(II) concentration and regulating the expression of the CnrCBA efflux pump that exports the excess of metal ions from the periplasm.[56] This system includes an extra-cytoplasmatic function (ECF)-type sigma factor, namely *Cm*CnrH. Sigma factors are crucial regulators of gene expression that bind RNA polymerase and determine promoter recognition and DNA melting at the transcription initiation site.[57] Sigma factors that respond to surface stimuli belong to the ECF family.[58] *Cm*CnrX is a membrane-anchored protein with a periplasmic domain acting as a receptor for metal ions. The crystal structure of its soluble dimeric domain (Fig. 5A) reveals the presence of a dimer.[56a] Ni(II) and Co(II), the inducers of the signalling cascade, bind in an octahedral geometry (Fig. 5B), while Zn(II) binds in a trigonal bipyramidal environment and leads to an inactive form of the protein.[59] *Cm*CnrX interacts with the *trans*-membrane protein *Cm*CnrY, which is the anti-sigma factor that sequestrates CnrH in its cytoplasmatic side. The structure of the *Cm*CnrH in complex with the cytoplasmatic domain of CmCnrY is shown in Fig. 5C.[60] Binding of metal ions to *Cm*CnrX in the periplasm triggers the signalling cascade that goes through *Cm*CnrY to the release of *Cm*CnrH. The latter then can diffuse in the cytoplasm to the DNA molecule and positively regulates the synthesis of proteins involved in Co(II) and Ni(II) resistance.[56a]

Other Ni(II) sensors

Streptomyces griseus expresses a Fe(II)–Zn(II) superoxide dismutase, named SodF, whose synthesis is regulated by Ni(II) levels.[61] The sensor responsible for this transcriptional control is a two-component system, constituted by SrnR and SrnQ. SrnR is a DNA binding protein of the ArsR/SmtB family. SrnQ, on the other hand, does not show similarity with known proteins. Transcriptional regulation of *sodF* requires both proteins, which form a complex able to bind DNA in the presence of Ni(II), *in vitro*. A model was proposed that indicates that SrnQ is the nickel sensor, whose metal-driven conformational change enhances the DNA binding activity of SrnR through the protein–protein interaction.[62]

NimR from the pathogen *Haemophilus influenzae* is a regulator belonging to the MerR family.[63] NimR regulates the expression of a Ni(II) uptake transporter (NikKLMQO). The promoters for *nimR* and the *nik* operon are divergent and overlapping. NimR responds to Ni(II) and to acidic pH, and binds at a site within the promoter elements for *nikKLMQO*.[63] Unusually for MerR-like regulators, NimR regulates the uptake of Ni(II) into the cell and activates transcription in the apo-form, while it acts as a repressor when bound to nickel. Purified NimR is a dimer that binds one Ni(II).[63]

In *Leptospirillum ferriphilum* UBK03 a novel Ni(II)-dependent transcription factor named NcrB was found to regulate both the expression of the membrane-associated NcrAC efflux system responsible for Ni(II) resistance, as well as its own expression.[64] In this system, Ni(II) acts as a co-repressor. No structural or biochemical data are available for this protein.

Finally, a urease control system has been recently identified in *H. pylori*, in addition to the well-established NikR-based network: the Ni(II)-responsive transcription factor Mua appears to repress urease activity at high Ni(II) concentrations.[65] The protein, which binds two Ni(II) per dimer with half saturation at 20 μM, does not bind directly the urease promoter, and it was thus suggested that it regulates urease expression through an uncharacterized indirect mechanism.[65]

Perspectives

The study of metal-dependent gene regulation can be considered the new frontier of bio-inorganic chemistry. It requires a complete clarification of the ways in which metal ions bind to transcriptional regulators, and of the selectivity processes that allow protein to specifically respond to the intracellular metal ion pool. Therefore, this understanding entails a full grasp of metal ion coordination chemistry, molecular biology, biochemistry, cell biology, structural biology, and several biophysical methods, which correlate metal ion availability to metal binding and protein functional responses. In the case of Ni(II), affinity in the nanomolar to low-micromolar range has been consistently detected for many characterized metal sensors, as described above. This result is compatible with an average intracellular Ni(II) concentration in this range, which, in *E. coli*, corresponds roughly to 10–500 Ni(II) ions per cell.[1a] This observation renders unlikely values of affinities of sensor proteins in the picomolar range, which would correspond to less than one metal ion per single cell in *E. coli*,[1a] as proteins with such affinities would be continuously loaded with metal ions, therefore unable to switch on or off the metal homeostasis systems. Considering this context and the relatively small number of nickel-containing proteins as compared to copper-, iron- or zinc-proteins, nickel-dependent gene regulation is an ideal prototypical case study.[1c]

In conclusion, understanding the determinants of intracellular metal homeostasis represents a novel challenge both for researchers with a chemical background, aiming to explore the chemistry of metal ions in biological systems, as well as for biochemists or molecular biologists eager to widen their knowledge in the inorganic chemistry of metals that are essential for life as we know it. In most cases, these two perspectives require a closely entangled multi-disciplinary approach that could induce different research groups to undergo a synergic effort to unravel this new fascinating side of biology.

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