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Structural determinants of metal selectivity in prokaryotic metal-responsive transcriptional regulators

Mario A. Pennella & David P. Giedroc*
*Department of Biochemistry and Biophysics, Texas A&M University, 2128 TAMU, College Station, Texas, 77843-2128, USA; *Author for correspondence (Tel: 979-845-4231; Fax: 979-845-4946; E-mail: giedroc@tamu.edu)*

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Abstract

Metal ion homeostasis in prokaryotes is maintained by metal-responsive transcriptional regulatory proteins that regulate the transcription of genes encoding proteins responsible for metal detoxification, sequestration, efflux and uptake. These metalloregulatory, or metal sensor proteins, bind a wide range of specific metal ions directly; this in turn, allosterically regulates (enhances or decreases) operator/promoter binding affinity or promoter structure. Recent structural studies reveal five distinct families of metal sensor proteins. The MerR and ArsR/SmtB families regulate the expression of genes required for metal ion detoxification, efflux and sequestration; here, metal binding leads to activation (MerR) or derepression (ArsR/SmtB) of the resistance operon. In contrast, the DtxR, Fur, and NikR families regulate genes encoding proteins involved in metal ion uptake; in these cases, the metal ion functions as a co-repressor in turning off uptake genes under metal-replete conditions. Inspection of the structures of representative members from each metal sensor family reveals several common characteristics: (1) they function as homo-oligomers (either dimers or tetramers); (2) metal-binding ligands are found at subunit interfaces, with ligands derived from more than one protomer; this likely helps drive quaternary structural changes that mediate allosteric coupling between the metal and DNA binding sites; and (3) the primary determinant of metal ion selectivity within each protein family is dictated by the coordination geometry of the metal chelate, with trends consistent with expectations from fundamental inorganic chemistry. This review highlights recent efforts to elucidate the structure of metal sensing chelates and the molecular mechanisms of allosteric coupling in metal sensor proteins.

Transition metal ion homeostasis

The biological activities of one-third of all proteins require essential transition metal ions that perform catalytic, structural or regulatory functions (Rosenzweig 2002). Key transition metal ions include the first row elements zinc, copper, nickel, cobalt, iron and manganese, as well as second and third row elements molybdenum and tungsten, each with distinct biological roles. Although often required in trace amounts, suboptimal or toxic (above normal physiological levels) concentrations of intracellular metal ions have severe, often pleiotropic affects on many aspects of cellular metabolism. This necessitates that all cells, from bacteria to plants to other eukaryotes, must be capable of obtaining or scavenging trace metal ions from their environment to meet basic cellular needs; in addition, resistance mechanisms must be inducible when concentrations exceed physiological needs. This balance of metal ion uptake and resistance mechanisms in cells is termed metal ion homeostasis.

With the exception of iron homeostasis in mammalian cells, metal homeostasis in all organisms requires regulation at the level of transcription. Metalloregulatory or 'metal sensor' proteins bind metal ions directly and repress, derepress, or activate the transcription of operons that encode metal-specific efflux pumps and/or membrane-bound transporters, metal reductases, soluble cytoplasmic or periplasmic metal transport proteins, or metal-sequestering proteins, as well as the metal-responsive transcriptional regulator itself (Silver & Phung 1996). This ensures that the cytosol, and in the case of eukaryotic cells, organelles, have the proper complement of transition metal ions (Blencowe & Morby 2003; Eide 2003). Not surprisingly, analogous and often overlapping, transcriptional regulatory mechanisms are also used by cells to mount a response to heavy metal pollutants that have no biological function, e.g., arsenic, mercury, cadmium and lead.

Arguably the most remarkable feature of metal homeostasis is that specific metal sensor proteins must be capable of discriminating among a number of superficially identical 'ligands', i.e., transition metal ions that often have similar sizes (ionic radii) and net charges (often 2+). Recent work reviewed here reveals that this challenging problem in molecular recognition is elegantly solved by the distinct coordination chemistries of specific 'metal receptor sites in metal sensor proteins. Proteins bind metal ions nearly exclusively via coordination by three types of atoms, sulfurs (Cys and Met), nitrogens (His), and oxygens (Asp and Glu). Metal ion binding sites in proteins adopt a variety of coordination or 'chelate structures. The primary coordination shell of donor ligands is defined by the number of donor atoms (coordination number), the coordination geometry (dictated by the coordination number, a set of metal-ligand bond lengths, and ligand-metal-ligand dihedral angles), and the types of ligands that coordinate the metal. These specific features, in turn, influence the number and nature of second coordination shell interactions (Frausto da Silva & Williams 2001). Typical coordination numbers (n) for transition metals in biological systems range from three to six, with coordination geometries ranging from trigonal ($n = 3$), to tetrahedral, square planar and trigonal pyramidyl ($n = 4$), to trigonal bipyramidal ($n = 5$), to octahedral ($n = 6$). Specific metal ions have clear preferences regarding coordination number and chelate geometries, as well as ligand types (Frausto da Silva & Williams 2001), and these preferences govern metal selectivity in biological systems.

Metalloregulatory proteins

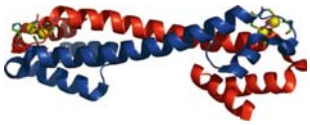
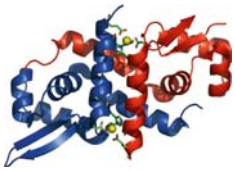
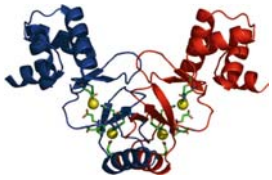
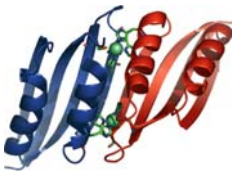
Through extensive biochemical, molecular genetic and more recently, whole genome sequencing and structural biology efforts, a large number of known or putative metal-responsive regulatory proteins have been identified, and extensively characterized. Five structural families of prokaryotic metal sensing transcriptional regulators emerge from these studies (Table 1). They include the MerR, ArsR (or ArsR/SmtB), DtxR, Fur, and NikR families of metalloregulators. Structurally related metal-specific regulators that sense distinct metal ions have been discovered in four of the five protein families listed in Table 1 (as of late 2004, the NikR family contains only nickel-specific regulators). For example, zinc-specific metal sensor proteins have been characterized in the MerR (ZntR), ArsR (SmtB and CzrA) and Fur (Zur) families. In this review, the structures of representative members of individual prokaryotic metal sensor families are discussed in turn. Emphasis is placed on how individual members from the same family recognize or detect a specific metal ion, e.g., zinc *versus* other metal ions, and how metal binding positively or negatively allosterically regulates operator/promoter DNA binding and transcription of the resistance/uptake operons.

Regulators of metal detoxification, efflux and sequestration

MerR family

The MerR family of regulators activate gene expression by distorting the operator DNA structure when bound to their cognate metal ion; this allows RNA polymerase to initiate transcription from an otherwise suboptimal promoter (Brown *et al.* 2003). Members of this family are characterized by N-terminal winged-helix DNA binding domains, followed by a long coiled-coil region which doubles as the primary dimer interface (Table 1). A small C-terminal helical domain is the effector binding domain (Brown *et al.* 2003). MerR

Table 1. Families of metalloregulatory proteins in prokaryotes.

Family	Fold/DNA binding motif	Members(s)[a]	States[b]	Structure[c]
MerR	$\beta\alpha\alpha\beta\beta\alpha\alpha\alpha$/N-terminal winged helix	MerR (Hg) CueR (Cu) ZntR (Zn) PbrR (Pb)	apo: weak repressor metallated: activator	
ArsR/SmtB	$\alpha\alpha\alpha\alpha\beta\beta\alpha$/winged HTH	ArsR (As, Sb) CadC (Cd, Pb) SmtB, CzrR (Zn) NmtR (Ni) BxmR (Cu,Zn)	apo: repressor metallated: low affinity	
DtxR	$\alpha\alpha\alpha\beta\beta\alpha\alpha\alpha\alpha$/N-terminal winged helix	DtxR (Fe) IdeR (Fe) MntR (Mn)	apo: low DNA affinity metallated: repressor	
Fur	$\alpha\alpha\alpha\alpha\beta\beta\beta\beta\beta\alpha\beta$/N-terminal winged helix	Fur (Fe) Zur (Zn)	apo: low DNA affinity metallated: repressor	
NikR	$\beta\alpha\alpha\beta\alpha\beta\beta\alpha\beta$/N-term ribbon-helix-helix	NikR (Ni)	apo: low DNA affinity metallated: repressor	

[a]Select family members and the predominant metal(s) sensed *in vivo* and *in vitro*. [b]Functional states of the apo- and metallated forms of the regulators. [c]Representative crystal structures from members of each family: MerR, Zn(II)-ZntR (Changela *et al.* 2003); ArsR, Zn_2 SmtB homodimer (Eicken *et al.* 2003); DtxR, Mn(II)-bound wild-type MntR (Glasfeld *et al.* 2003); Fur, Zn(II)-bound *P. aureginosa* Fur (Pohl *et al.* 2003); NikR, C-terminal tetramerization domain of Ni(II)-bound *E. coli* NikR (just two of the four protomers are shown) (Schreiter *et al.* 2003). For each structure, one protomer is colored blue and the other protomer is red. Small filled spheres in the structures represent bound metal ions. [d]Protein families that regulate genes required for metal export, sequestration and detoxification (MerR and SmtB/ArsR families) are listed first, followed by metal sensors that regulate metal uptake and assimilation (DtxR, Fur, and NikR families). See text for details.

orthologs that bind a wide range of inducers including metal ions, lipophilic drugs, nitric oxide and superoxide (induced by oxidative stress) are known. The paradigm of this family and hence where the name derives comes from the seminal studies of *merR* genes of transposons Tn*501* from *P. aeruginosa* and Tn*21* from *Shigella flexneri* R100 plasmid (Barkay *et al.* 2003). In gram-negative systems, MerR regulates expression of the mercury resistance divergon (*merTP(C/F)AD(E)* and *merR*) from the major regulated promoter. Regulation by MerR is discussed in more detail by Hobman, Wilkie, and Brown in this issue of Biometals.

Although the X-ray crystallographic structure of Hg(II)-MerR has not yet been solved, the

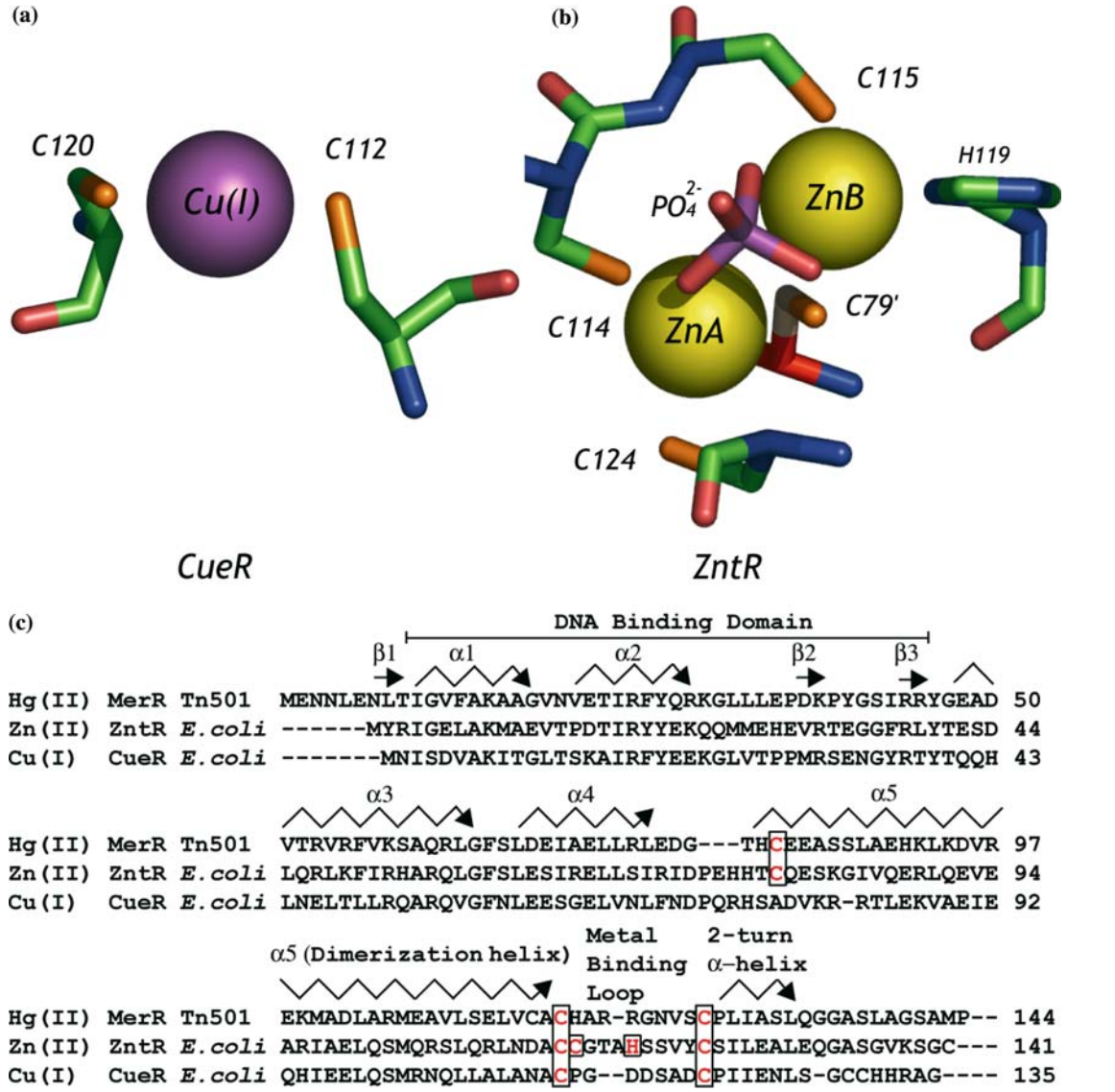


Figure 1. MerR family metalloregulators. (a) Close-up of the coordination chelate of CueR with bound Cu(I) shown as a purple sphere ligated to two cysteine residues from the same monomer (C112 and C120) to form a linear two-coordinate geometry (Changela *et al*. 2003). (b) Close-up of the metal binding domain of ZntR with two bound zinc ions shown as *yellow* spheres. Four zinc-ligating residues are derived from one protomer (C114, C115, H119, and C124), while a phosphate (or sulfate) anion and the side chain of C79 from the other protomer bridge the two zinc ions. (c) Multiple sequence alignment of Hg(II)-specific *Tn501* MerR, Zn(II)-specific *E. coli* ZntR, and Cu(I)-specific *E. coli* CueR (Changela *et al*. 2003). Known metal binding residues are colored *red* and are boxed. Secondary structure elements are represented above the sequence: *β* strands, horizontal arrows; α-helices, zig-zag arrows.

structures of BmrR and MtaN, two MerR orthologs that regulate multi-drug resistance in pathogenic bacteria have been determined (Godsey *et al*. 2001; Heldwein & Brennan 2001). These studies were the first to define the basic architecture of a MerR-family homodimer (see Table 1), as well the molecular details of promoter 'remodeling' by a drug-BmrR complex (Heldwein & Brennan 2001). More recently, the crystal structures of two *E. coli*-derived metalloregulators from the MerR family have been reported (Changela *et al*. 2003). These include the zinc-specific regulator ZntR (Table 1) to 1.9 Å resolution, and CueR, a Cu(I) sensor to 2.2 Å resolution,

which regulates the expression of the efflux pump CopA and periplasmic copper oxidase CueO in response to moderate copper stress (Outten *et al.* 2000; Rensing & Grass 2003). Au(I)- and Ag(I)-liganded complexes of CueR (to 2.5 and 2.1 Å resolution, respectively) have also been determined (Changela *et al.* 2003). The structures provide molecular insight into the distinct metal coordination environments of ZntR *versus* CueR and thus the likely basis for metal specificity of MerR proteins.

E. coli ZntR is mainly zinc-inducible and regulates the expression of *zntA*, which encodes a Zn(II)/Cd(II)/Pb(II) ATPase. The ZntR operator is uniquely characterized by a 20 bp spacer between the canonical −35 and −10 regions of the promoter. ZntR exists as a homodimer, binds two zinc ions per monomer or four per dimer (as determined by X-ray crystallography), and utilizes a Hg(II)–MerR-like DNA distortion mechanism to activate the expression of *zntA* (Outten *et al.* 1999; Changela *et al.* 2003). Strikingly, X-ray crystallography reveals a binuclear zinc site with each Zn(II) ion bound in a tetrahedral coordination environment and an internuclear Zn–Zn distance of 3.6 Å (Changela *et al.* 2003) (Figure 1b). One zinc ion (designated as A) is ligated by Cys114 and Cys124 of the metal binding loop while the other zinc ion (designated as B) is ligated by Cys115 and His119 of same metal binding loop (Figure 1) (Changela *et al.* 2003). The two zinc ions are bridged by oxygen atoms of a phosphate (or sulfate) anion and by the sulfur atom of Cys79' from the other protomer.

This binuclear Zn(II) coordination complex in ZntR, coupled with the bound anion, was totally unexpected since Hg(II)–MerR requires just one trigonal planar Hg(II) ion that bridges the MerR dimer to effect metalloregulation of the *mer* operon (Utschig *et al.* 1995). The extent to which occupancy of one or both metal sites in each of the two symmetry-related metal-binding loops in ZntR regulates transcriptional activation has yet to be established, although substitution of all five protein-derived zinc ligands reduces or abrogates the Zn(II) response *in vivo* (Khan *et al.* 2002). Interestingly, inspection of the ZntR structure and sequence alignment of known ZntRs suggests that a $S_3N(O)$ mononuclear, tetrahedral Zn(II) complex involving Cys114, His(Asp)119, Cys124 and Cys79' could also be readily accommodated by ZntR. Three of the four metal binding cysteines in ZntR (Cys79', Cys114, and Cys124) are conserved in Hg(II)-sensing MerRs, and likely constitute the donor atoms to the subunit-bridging *tris*-thiolato Hg(II) complex (Utschig et al., 1995). In Cu(I)–CueR, cysteines analogous to Cys114 and Cys124 form an unusual linear two-coordinate S–Cu–S regulatory complex (Figure 1). Only in known zinc-sensing ZntRs are His29, His53 [which likely plays a role in mediating DNA distortion by Zn(II)–ZntR (Khan *et al.* 2002)] and His119 invariant or conserved. Hence, the metal selectivity of MerR family members derives from differences in coordination number and type of metal ligands utilized by different proteins (see Figure 1), a finding analogous to that previously demonstrated for two members of the ArsR/SmtB family, the zinc-sensing *S. aureus* CzrA *versus* the nickel-sensing NmtR (Pennella *et al.* 2003) (*vida infra*).

As a further test of the inducer-specificity of the metal-binding domains in MerR proteins, a chimeric protein in which the N-terminal winged-helix DNA-binding domain (44 residues) of *Tn501* MerR is fused to the C-terminal 103 amino acids of ZntR has been characterized. This MerR/ZntR chimera was found to activate transcription from a *mer* operator/promoter that contained a 20 bp −10/−35 spacer uniquely characteristic of the *zntA* promoter rather than the wild-type 19-bp spacer in the *mer* operon (Brocklehurst *et al.* 1999). Furthermore, activation was selective for Zn(II), consistent with the hypothesis that the C-terminal metal-binding domain of MerR proteins confers metal selectivity and adjustment of the appropriate degree of metal-induced DNA distortion required by various operator-promoter sequences (Brown *et al.* 2003).

ArsR (ArsR/SmtB) family

The ArsR/SmtB family consists of homodimeric 'winged' helix-turn-helix (HTH) (see Table 1) transcriptional repressors that are specifically bound to their operator/promoter (O/P) DNA binding sites in the metal-free apo state. Metal binding allosterically negatively regulates DNA binding and derepresses transcription (Busenlehner *et al.* 2003). Most of the O/P sequences of this family contain one or two imperfect 12-2-12 inverted repeats but

little is known at the molecular level of how the aporepressor binds to these sites. Individual members of this family share between 25% and 50% amino acid sequence identity; in addition, a conserved helix-turn-helix DNA binding motif is the major determinant that characterizes members of this family.

The name of this protein family (Shi *et al.* 1994) is derived from its two founding members, ArsR from *E. coli* (Wu & Rosen 1993) and *Synechococcus PCC 7942* SmtB (Morby *et al.* 1993). ArsR is an arsenic/antimony-responsive repressor of the *ars* operon which encodes an arsenate reductase, as well as proteins required for metal export (Wu & Rosen 1993; Shi *et al.* 1994; Busenlehner *et al.* 2003). SmtB functions as a zinc-responsive repressor that represses transcription of a divergently transcribed operon containing both the *smtA* gene (encodes a class II metallothionein) and the *smtB* gene (Huckle *et al.* 1993; Morby *et al.* 1993; Busenlehner *et al.* 2003).

Metal binding sites of ArsR/SmtB proteins

Over 10 years of molecular genetic, biochemical, biophysical, and structural studies revealed that this family of metal-sensing transcriptional repressors generally possess at least two structurally distinct metal binding sites (Busenlehner *et al.* 2003). They are termed the α3N site and the α5 site (see Figure 2B). The α3N site is composed of cysteine residues from the N-terminal portion of the protein and a highly conserved ELCVCD motif (located at the α3 helix), initially termed the 'metal binding box' (Busenlehner *et al.* 2003). The α5 site is composed of histidines and carboxylate residues, and is found in the C-terminal portion of the protein (see Table 1). Some ArsR/SmtB regulators possess the α3N site only, others the α5 site only, some have both sites (Busenlehner *et al.* 2003), and a few have neither (at least some of these regulators possess a structurally distinct Cd(II)/Pb(II) metal binding site) (Cavet *et al.* 2003). The α5 site interacts preferably with smaller divalent metal ions, e.g., Zn(II), Co(II), and Ni(II), while the α3N site preferentially binds larger, more thiophilic heavy metal pollutants such as Cd(II), Pb(II), and Bi(III). ArsR/SmtB proteins that possess both metal binding sites have been shown to utilize only one site for metalloregulation, but typically not both (Busenlehner *et al.* 2002; VanZile *et al.* 2002). It will be interesting to determine if this is indeed the case for a recently discovered Cu(I)/Zn(II) sensor, *Oscillatoria brevis* BxmR; this ortholog may well utilize different sites to effect regulation by different metal ions (Liu *et al.* 2004). What factors determine which metal binding site is functional when both are present remains unclear, but recent work suggests that the metal selectivity of the allosteric response requires determinants outside of the primary coordination shell (Eicken *et al.* 2003; Pennella *et al.* 2003).

α5 sensors: CzrA, NmtR, and SmtB

Staphylococcus aureus CzrA is a Zn(II)/Co(II) sensing repressor of the *czr* (chromosomal zinc regulator) operon. Like SmtB, CzrA metalloregulates DNA-binding through zinc-binding to the α5 site (Eicken *et al.* 2003; Pennella *et al.* 2003). Structural studies of the apo- and Zn(II)-forms of CzrA and SmtB reveal that the α5 metal binding site is formed by a pair of ligands derived from the α5 helix of one protomer, D84 (D104 in SmtB) and H86 (H106), and two derived from the α5 helix of the other protomer, H97' (H117') and H100' (E120') (Eicken *et al.* 2003) (Figure 2a). The structure of Zn(II)-bound SmtB reveals an intersubunit hydrogen-bonding network that appears to link the metal binding site (α5) to the putative DNA binding helix (αR), via just two successive side chain (His117 N2)-main chain (R87' O), main chain (L88' αNH)-main chain (αR L83' O) hydrogen bonds (Eicken *et al.* 2003). Formation of these hydrogen bonds drives a quaternary structural conformational switch that compacts the homodimer and changes the relative disposition of the two αR reading heads, likely moving them out of register with respect to successive major grooves of the duplex DNA. Hydrogen-deuterium exchange studies carried out with *S. aureus* CzrA by NMR methods are consistent with the formation of this hydrogen bonding network in solution as well, with zinc binding to the α5 sites dramatically dampening internal dynamics throughout the dimer (Eicken *et al.* 2003). This work provides the first clear evidence of how metal binding allosterically regulates DNA binding in a prokaryotic metalloregulator at the atomic level.

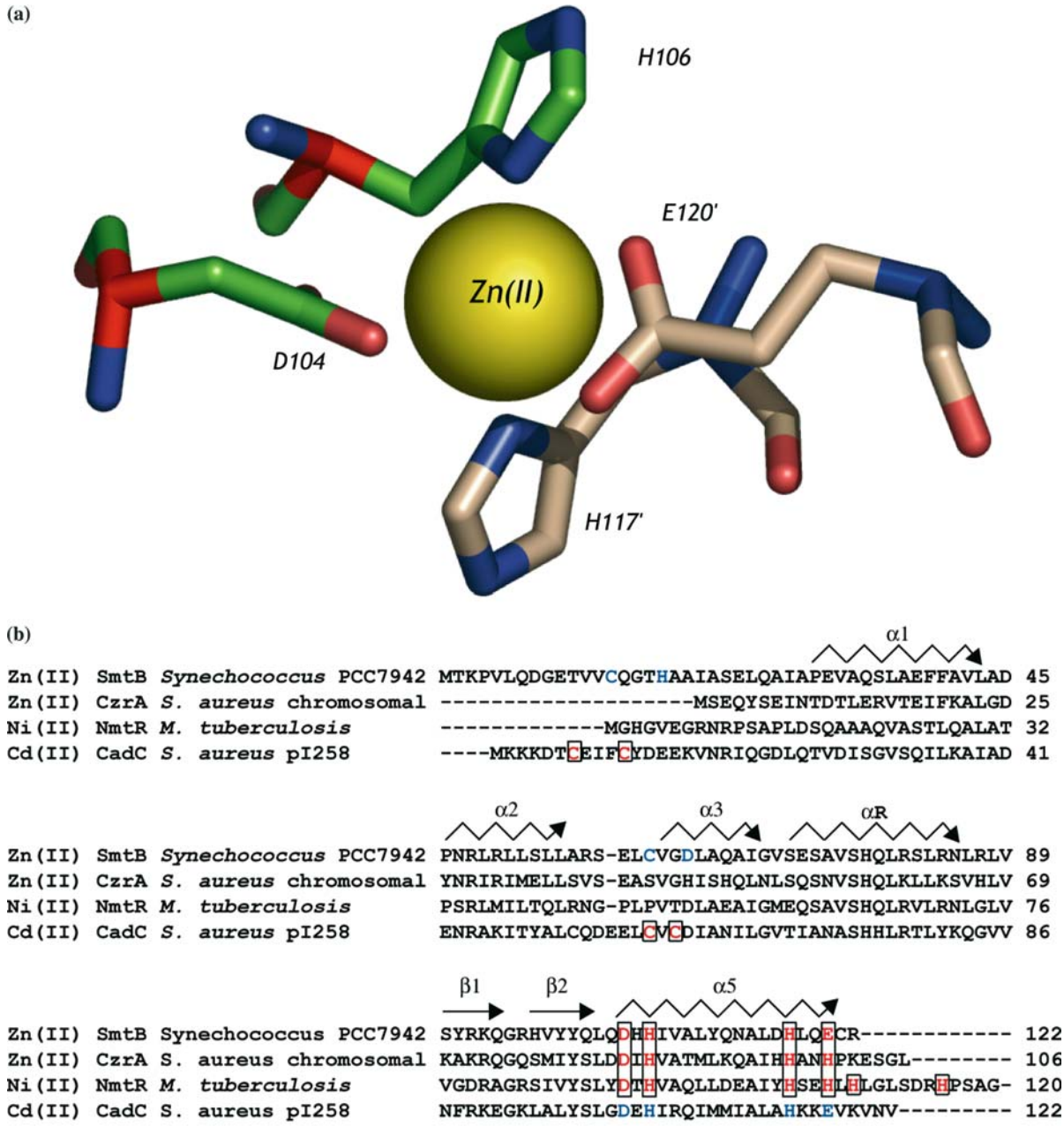


Figure 2. ArsR/SmtB family metalloregulators. (a) Close-up of the coordination chelate of Zn_2 SmtB homodimer (Eicken *et al.* 2003). Zinc ion is represented as a *yellow* colored sphere. Two zinc ligating residues derived from one protomer (D104 and H106) and two (H117 and E120) from the other protomer coordinate the zinc ion in a tetrahedral coordination geometry. (b) Multiple sequence alignment of *Synechococcus* SmtB, *S. aureus* CzrA, *M. tuberculosis* NmtR, and *S. aureus* pI258 CadC. Known metal binding residues are boxed (Busenlehner *et al.* 2003). Residues shown to be involved in negatively regulating DNA binding are colored *red*. *Blue* residues bind metal ions but are *not* involved in metalloregulation. α3N amino acids are the boxed and *red* cysteines in CadC. Secondary structural elements are based upon the crystal structure of SmtB and CzrA (Eicken *et al.* 2003) (see Table 1).

CzrA coordinates Zn(II) and Co(II) with tetrahedral geometry while Ni(II), a non-inducing metal ion, adopts a five or six-coordinate geometry (Pennella *et al.* 2003). Strikingly, in the case of the Ni(II)/Co(II) sensing repressor *Mycobacterium tuberculosis* NmtR, optical and X-ray absorption

spectroscopies reveal Co(II) and Ni(II) bind with five or six-coordinate geometries, while the poorly-functional Zn(II) ion binds with four-coordinate geometry (Cavet *et al*. 2002; Pennella *et al*. 2003). Thus, NmtR expands the coordination number of the α5 metal binding site to form a five- or six-coordinate complex, currently thought to occur via recruitment of two additional histidine ligands found C-terminal to the α5 helix (Figure 2B). This effectively switches the metal selectivity of an α5 sensor from Zn(II)/Co(II) to Ni(II)/Co(II). Note that Co(II) is an effective regulator of both CzrA and NmtR, due to the fact that Co(II) is 'on the fence' relative to coordination number preferences, unlike Zn(II) ($n = 4$) and Ni(II) ($n = 6$); Co(II) will often adopt the coordination geometry enforced by the protein structure. Another important finding is that the Ni(II)/Co(II) regulator NmtR binds Zn(II), a weaker allosteric regulator, with an affinity that is equivalent to, or higher than, Ni(II) (Cavet *et al*. 2002). These and other data argue (Cavet *et al*. 2002) that the metal that binds with the highest stability does not always result in strong metalloregulation *in vivo* or *in vitro*. These results collectively reveal that metal coordination number and geometry are primary determinants of biological metal specificity (Cavet *et al*. 2002; Pennella *et al*. 2003).

Regulators of metal ion uptake

DtxR family

The DtxR family comprises a group of largely iron-dependent metalloregulators and is named for its founding member, the diptheria toxin repressor (DtxR) from *Cornybacterium diphtheria*. DtxR and a homolog found in *M*. *tuberculosis*, IdeR, function as Fe(II)-dependent repressors that bind to a hyphenated palindromic sequence as a dimer of dimers (Andrews *et al*. 2003). DtxR negatively regulates genes required for iron acquisition, and expression of a major virulence factor, diphtheria toxin, in response to its co-repressor iron. IdeR is encoded by an essential gene in *Mycobacterium* (Rodriguez *et al*. 2002) and not only plays roles in iron assimilation and storage, but also appears to promote survival in macrophages by regulating an oxidative stress response (Gold *et al*. 2001). DtxR/IdeR regulated genes are constitutively expressed when iron is limiting in the environment, which is typically the case. Direct sensing of cytoplasmic Fe by DtxR/IdeR represses the expression of iron uptake and virulence genes thereby mediating iron homeostasis in these pathogenic bacteria.

DtxR/IdeR contains an N-terminal winged-helix (ααααββα) DNA binding domain followed by a helical dimerization domain; some DtxRs also contain a C-terminal Src homology 3 (SH3)-like domain that is rare in bacterial proteins (White *et al*. 1998) (see Table 1). At least 18 crystallographic structures of *C*. *diphtheria* DtxR and *M*. *tuberculosis* IdeR have been reported with a variety of divalent metal ions bound, in the presence and absence of DNA (*cf*. (White *et al*. 1998). While the coordination structures and functional importance of these bound metal ions is controversial (see Chou *et al*. 2004), two distinct metal sites in DtxR/IdeR, termed site 1 and site 2, emerge clearly from these studies (Table 1). Recent work suggests that metal site 1, somewhat distal from the DNA binding domain, stabilizes the IdeR dimer, thereby facilitating the binding of metal ion to site 2, which activates DNA binding (Chou *et al*. 2004). Metal site 1 adopts a distorted trigonal bipyramidal pentacoordinate geometry and is liganded by three residues derived from the α4 (H79, E83) and α5 (H98) helices (see Figure 3c), and two residues contributed by the C-terminal SH3 domain (E170 and Q173). Metal site 2, the putative metalloregulatory site, adopts a distorted five (DtxR)- or six (IdeR)-coordinate octahedral site formed by three closely spaced and invariant residues in the α5 helix (C102, E105, and H106) as well as M10 from helix α1 (Figure 3c) (White *et al*. 1998). The mechanism of allosteric positive regulation of operator/promoter binding by DtxR appears to involve a hinge-binding motion of the DNA-binding domains with respect to the dimerization domain of DtxR. This metal-mediated quaternary structural change moves the DNA-binding helices by 3 –5 Å when compared to the aporepressor, allowing them to fit snugly into successive major grooves on the duplex DNA (Pohl *et al*. 1999).

DtxR (IdeR) orthologs have recently been discovered that regulate the expression of genes encoding metal transporters that are apparently specific for other divalent metal ions, including Mn(II) and perhaps Zn(II). These include *Bacillus subtilis* MntR and *Treponema pallidum* TroR (Hazlett *et al*. 2003). *B*. *subtilis* MntR regulates the

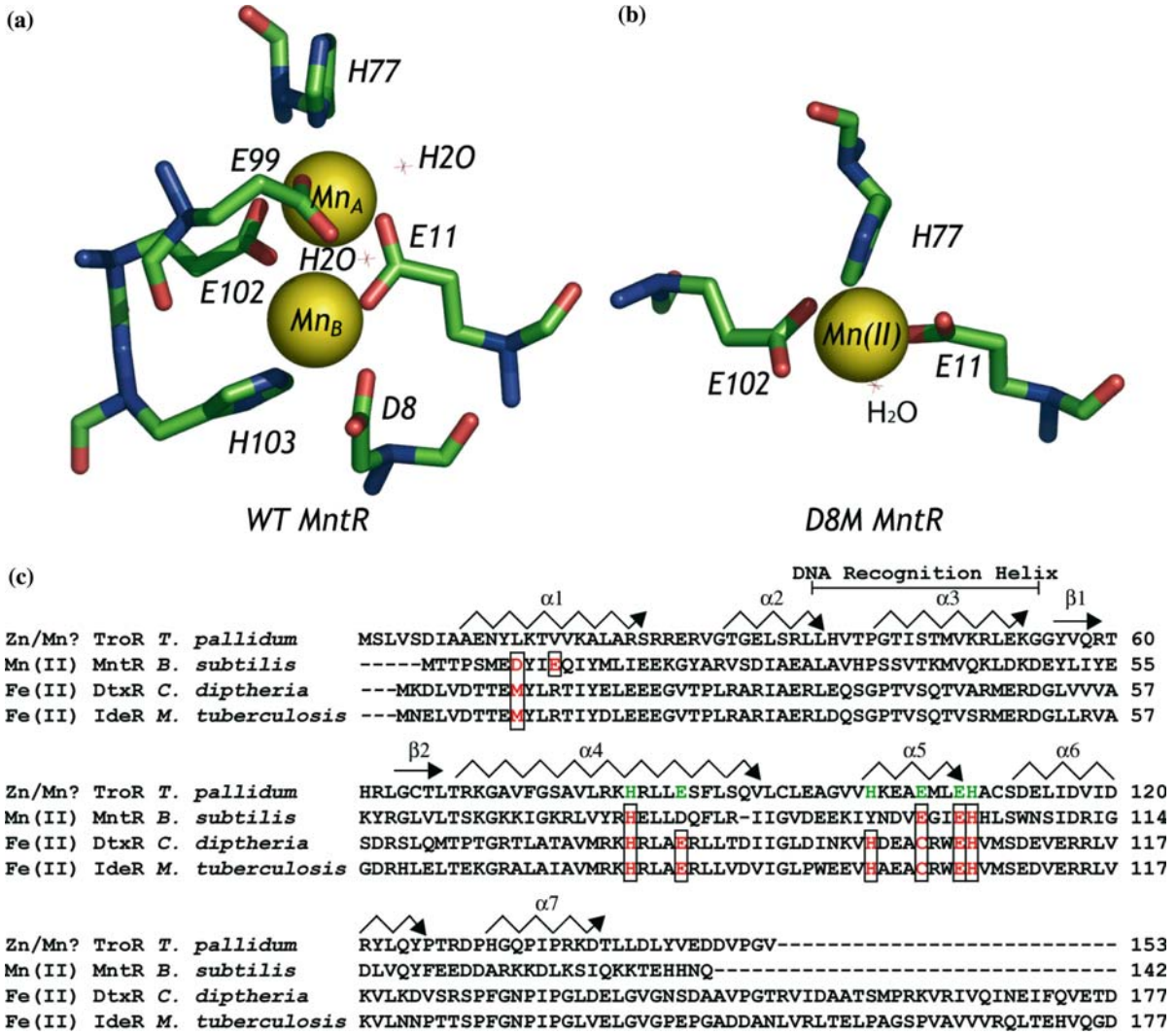


Figure 3. DtxR family metalloregulators. (a) Close-up of the metal binding domain of wild-type *B. subtilis* MntR with two Mn(II) ions (shown as *yellow* spheres) bound per protomer in a binuclear cluster. Residues E11, H77, E99, E102, and a solvent molecule (designated as small red xs and labeled H_2O) coordinate the Mn(II) ion designated as Mn_A while D8, E11, E102, and H103 coordinate the Mn(II) ion designated as Mn_B. The Mn(II) ions are bridged by bidentate interactions of E11, E102, and another solvent molecule. (b) The metal coordination chelate of D8M MntR with one Mn(II) bound per protomer. Residues E11, H77, and E102 coordinate the Mn(II) ion along with a solvent molecule. (c) Multiple sequence alignment of *T. pallidum* TroR, *B. subtilis* MntR, *C. diphtheria* DtxR, and *M. tuberculosis* IdeR with the secondary structure regions (based upon crystal structure of MntR) shown as above (Table 1). Known metal binding residues are colored *red* and boxed. Proposed metal binding residues of TroR are shaded *green*.

expression of two manganese transporters, the Nramp-type transporter MntH under high Mn(II) and the ABC-type transporter MntABCD under low Mn(II) conditions (Glasfeld *et al*. 2003; Kehres & Maguire 2003). In high manganese, Mn(II)–MntR functions as a repressor by binding to operators upstream of the coding regions of both *mntH* and *mntABCD* operons. MntR is selective for manganese while DtxR is activated to bind DNA by various divalent metals including manganese *in vitro* (Ikeda *et al*. 2005).

Strikingly, the high resolution crystallographic structure of Mn(II)-bound MntR (to 1.75 Å resolution) reveals a binuclear Mn(II) center, with the two Mn(II) ions bridged by two glutamates, E102 and E11, the latter unique to MntR, and a solvent molecule (Figure 3A) (Glasfeld *et al*. 2003). The other ligands to the site A Mn(II) ion are H77 and

E99 (C102 in DtxR) and two water molecules, creating a distorted octahedral coordination geometry (Glasfeld *et al*. 2003). Mn_B also adopts an octahedral geometry and is ligated by D8, which is M10 in Fe(II)-sensing DtxR, H103, and two solvent molecules, in addition to the bridging ligands E11 and E102 (Glasfeld *et al*. 2003). Mn_B is roughly positioned in the same place as a Co(II) ion bound to metal site 1 in IdeR (Pohl *et al*. 1999).

In an effort to understand the molecular basis of metal ion discrimination between DtxR/IdeR and MntR, a D8M substitution mutant of MntR was functionally and structurally characterized. D8M MntR shows relaxed specificity for Mn(II) and is activated by both Fe(II) and Mn(II) *in vivo* (Guedon & Helmann 2003). The high resolution crystallographic structure of D8M MntR (to 1.6 Å resolution) reveals why (Table 1, Figure 3). D8M MntR retains just one of the two Mn(II) ions and is coordinated by E11, H77, E102, and a solvent molecule (the introduced M8 is *not* ligated to the metal) in a distorted trigonal bipyramidal geometry, closer to the position of Mn_A in wild-type MntR (Glasfeld *et al*. 2003). These findings suggest that metal selectivity in the DtxR family is again governed by the number and type of ligands bound to the metal, analogous to findings in the MerR and ArsR/SmtB family regulators. Interestingly, the amino acid sequence of the putative Zn(II) regulator *T. pallidum* TroR lacks all N-terminal α1-derived metal binding residues that play critical roles in DtxR/IdeR (M10) and MntR (D8, E11) (see Figure 3c); this might lower the metal coordination number to $n = 4$, thereby explaining its reported ability to respond to Zn(II) *versus* other divalent transition metal ions (Hazlett *et al*. 2003).

Fur family

E. coli Fur and homologs in other prokaryotes regulate the transcription of genes required for iron acquisition, including a host of distinct iron scavenging systems as well as the enzymes for siderophore biosynthesis (Andrews *et al*. 2003). In addition, the expression of key virulence factors in pathogenic bacteria is also regulated by Fur. The working model is that the iron-bound form of Fur binds to specific target sequences in the promoter of iron-regulated genes, collectively known as 'iron boxes', blocking access by RNA polymerase. Like the DtxR family regulators, iron functions as a co-repressor of the expression of those genes linked to iron acquisition; in iron-starved conditions, these genes are constitutively expressed.

Just one high resolution crystallographic structure of a Fur-family regulator has been solved, namely *Pseudomonas aeruginosa* Fur (PA Fur) complexed with Zn(II) (rather than iron) to 1.8 Å resolution (Pohl *et al*. 2003). A ribbon representation of the structure is shown in Table 1. As can be seen, the global structure of the Fur homodimer is superficially similar to that of DtxR; in fact the winged-helix DNA-binding domains superimpose with a backbone rmsd of 1.8 Å from helix α2-α4 through the β1-β2 hairpin. The dimerization domain of each protomer is an α/β domain consisting of a 3-stranded β-sheet topped by the C-terminal α5 helix. Dimerization results in the formation of a six-stranded β-barrel capped by an antiparallel coiled-coil formed by the α5 helices, in a manner clearly distinct from that of DtxR.

Two metal ions are bound per protomer for a total of four metal ions per dimer, designated Zn1 and Zn2. Zn1 is coordinated by residues located exclusively in the dimerization domain and adopts a distorted octahedral geometry with four protein-derived ligands (H86, D88, E107 and H124), including one bidentate carboxylate (D88) and a well-ordered water molecule (see Figure 4a). Zn2 bridges the DNA-binding and dimerization domains and adopts distorted tetrahedral coordination geometry (H32, E80, H89 and E100) (Pohl *et al*. 2003) (see Figure 4A). Fe(II) exchange experiments and characterization of the resulting Zn/Fe hybrid protein by X-ray absorption spectroscopy reveals that Zn1 is readily exchanged with Fe(II) and adopts a coordination complex that is isostructural with Zn1, with coordination of Zn2 unchanged. Therefore, the authors argue that in PA Fur, the regulatory metal site corresponds to Zn1 located exclusively in the dimerization domain, while Zn2 is an unusual structural Zn(II) site, which may form part of the long-range allosteric pathway of communication between the regulatory sites and the DNA-binding domains of PA Fur (Pohl *et al*. 2003). Although the mechanism of metal-dependent activation of Fur binding to DNA is still unknown, it seems plausible that metal binding to site 1 globally alters the orientation of one subunit relative to the other (as in the case of SmtB and DtxR), stabilizing a high-affinity

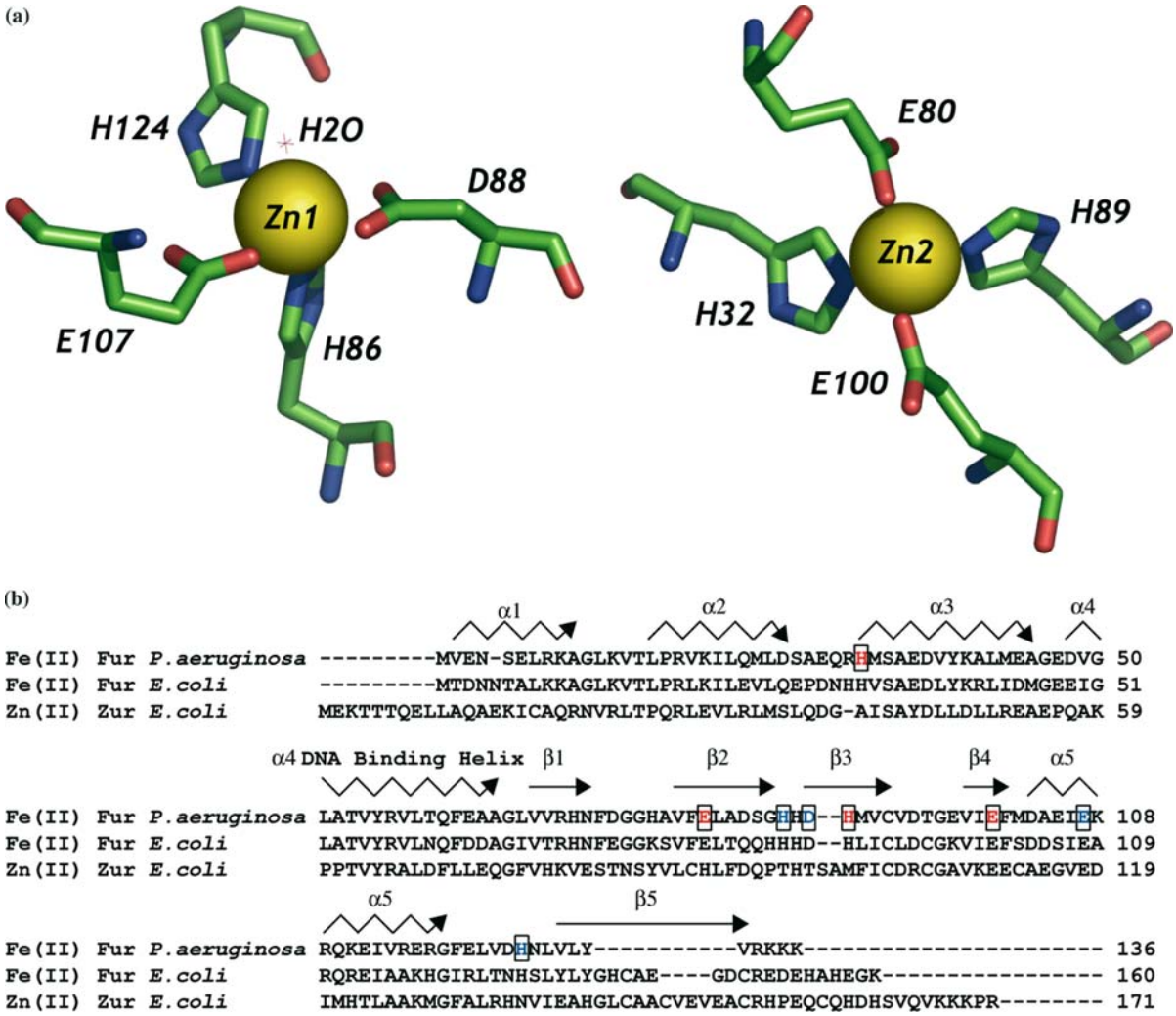


Figure 4. Fur family metalloregulators. (a) Close-up of the coordination chelates of the Zn_4 *P. aeruginosa* Fur homodimer. Zinc ions shown are represented as *yellow* spheres. Residues H86, bidentate D88, E107, H124, and an ordered water molecule coordinate Zn1. Residues H32, E80, H89 and E100 bind Zn2 with distorted tetrahedral coordination geometry. (b) Multiple sequence alignment of *P. aeruginosa* Fur, *E. coli* Fur, and *E. coli* Zur using ClustalW (http://www.ebi.ac.uk/clustalw) (Thompson *et al.* 1997). Known metal binding ligands are boxed and the secondary structural elements are defined as above (Table 1). *Blue* residues in *P. aeruginosa* Fur represent the proposed regulatory Fe binding site; *Red* residues in *P. aeruginosa* represent the proposed structural Zn binding site.

binding conformation. All Fur homologs contain the full complement of metal sites structurally characterized in PA Fur.

Interestingly, many Fur homologs, including *E. coli* Fur, contain two pairs of cysteine residues not present in PA Fur. One pair is C92 and C95, just C-terminal to H86 and D88 of the Zn1(Fe) site and H89 of the Zn2 site, and N-terminal to E100 of Zn2 site in PA Fur; PA Fur contains a residue analogous to C92 only which does not participate in metal binding (see Figure 4b). In addition, most Fur homologs contain a short C-terminal extension that contains a C–X_4–C sequence that is not present in PA Fur (Figure 4b). X-ray absorption studies of *E. coli* Fur suggest at least one sulfur-containing Zn(II) coordination complex; spectroscopic studies of mutants suggest that C92 and C95 ligate this Zn(II) ion in the formation of a structural $S_2(N/O)_2$ complex, although this remains to be definitely determined (Jacquamet *et al.* 1998; Gonzalez de Peredo *et al.* 1999).

E. coli Zur is a recently discovered Fur ortholog (27% sequence identity) that regulates the expression of a divergently transcribed operon that

encodes the high affinity zinc uptake system, *znuBC* and *znuA*, in a highly Zn(II)-specific fashion (Patzer & Hantke 1998, 2000). The high resolution structure of Zur is not yet available and the molecular basis for metal ion discrimination by *E. coli* Zur remains unclear. However, it is possible to speculate on metal selectivity on the basis of an amino acid sequence alignment and limited spectroscopic findings reported for the Zn(II) and Co(II) metalloderivatives of *E. coli* Zur. Zur binds two metal ions per subunit and four per dimer in two spectroscopically distinguishable tetrahedral sites (Outten *et al.* 2001). One Zn(II) ion, ZnA, is tightly bound in an $S_3(N/O)$ coordination environment and is refractory to metal exchange with Co(II). This site is likely analogous to the structural Zn2 site in Fur homologs that contain the C92–X_2–C95 cysteine pair. The other site, ZnB, is readily exchanged with Co(II) and is characterized by tetrahedral $S(N/O)_3$ coordination geometry, in striking contrast to the octahedral, putative Fe(II)-sensing Zn1(Fe) site of PA Fur (Outten *et al.* 2001). Although the four donor atoms to this putative Zn(II)-sensing site remain unknown, all functionally characterized Zur proteins conserve at least two cysteines just C-terminal to the putative α5 helix in *E. coli* Zur (Figure 4b); one of these may well correspond to the thiolate donor in the ZnB site. Regardless of the details, these comparative structural studies of Fur and Zur emphasize the recurring theme that metal selectivity in metal sensor proteins is dictated by coordination number ($n = 6$ for Fur; $n = 4$ for Zur) and geometry, with a lesser role played by the type of the donor atoms. This is nicely in accord with the conclusions reached by the Fierke group from a careful investigation of the metal affinities and structural studies of a extensive panel of carbonic anhydrase mutants (McCall & Fierke 2004).

NikR family

NikR is a metal-responsive transcriptional regulator that mediates the repression of the *nik* operon (*nikABCDE*) in gram-negative bacteria and archaea in response to Ni(II). The *nik* operon encodes a high affinity nickel specific permease that belongs to the ABC transporter family (Navarro *et al.* 1993; De Pina *et al.* 1999). NikR contains a ribbon–helix–helix motif, and has been shown to be a homotetramer in the presence and absence of Ni(II) (Chivers & Sauer 1999, 2002). NikR binds an operator sequence within the *nik* promoter with high affinity under conditions of saturating Ni(II) in the cell (Chivers & Sauer 2002). The apo-form of NikR has not been shown to bind DNA, and it is likely that it does not. The crystallographic structure of apo-NikR reveals that the dimeric ribbon-helix-turn-helix domains responsible for DNA binding are on distal ends of the tetrameric C-terminal regulatory domain, each of which likely interacts with a pair of dyad-symmetric half-sites 5′-GTATGA-3′ on opposite ends of an imperfect 16-6-16 inverted repeat (Schreiter *et al.* 2003) (see Table 1). NikR is therefore formally a dimer of dimers.

There are two distinct classes of Ni(II) binding sites on tetrameric NikR. One class of sites exhibits a very high affinity for Ni(II) (a Ni(II) dissociation equilibrium constant in the pM range) and activates the binding of the NikR tetramer to ~40 base pairs of operator DNA with ~nM DNA binding affinity. The other Ni(II) binding site(s) comprise lower affinity sites (30 nM to low μM dissociation constants) that further expand the DNA binding footprint of NikR from 40 bp to ~65 bp, and enhance the DNA binding affinity of NikR into the pM range (Chivers & Sauer 2000, 2002; Carrington *et al.* 2003). The structures and locations of these site(s) on the NikR tetramer are completely unknown (But in recent paper describes crystallographic structures of various forms of pyrococcus horikoshii NikR, two of which contain Ni(II) bound to the putative "low-affinity" sites (Chivers PT, Tahirov TH. 2005 J Mol Biol 248, 597–607) These structures further suggest a model for Ni(II)-dependent activation of high affinity DNA binding). In contrast, the coordination geometry of the high affinity Ni(II) site has been determined for the free (not complexed to DNA) full-length protein as well as the C-terminal regulatory domains (residues 48–133; this is the metal binding and tetramerization domain). In both cases, Ni(II) adopts a relatively rare, square planar coordination geometry via coordination by one thiolate and three imidazole ligands (Carrington *et al.* 2003; Schreiter *et al.* 2003) and is bound within successive protomer interfaces around the tetramerization domain (see Table 1). As found in the other metal sensor families, the high affinity Ni(II) site is formed by ligands derived from different

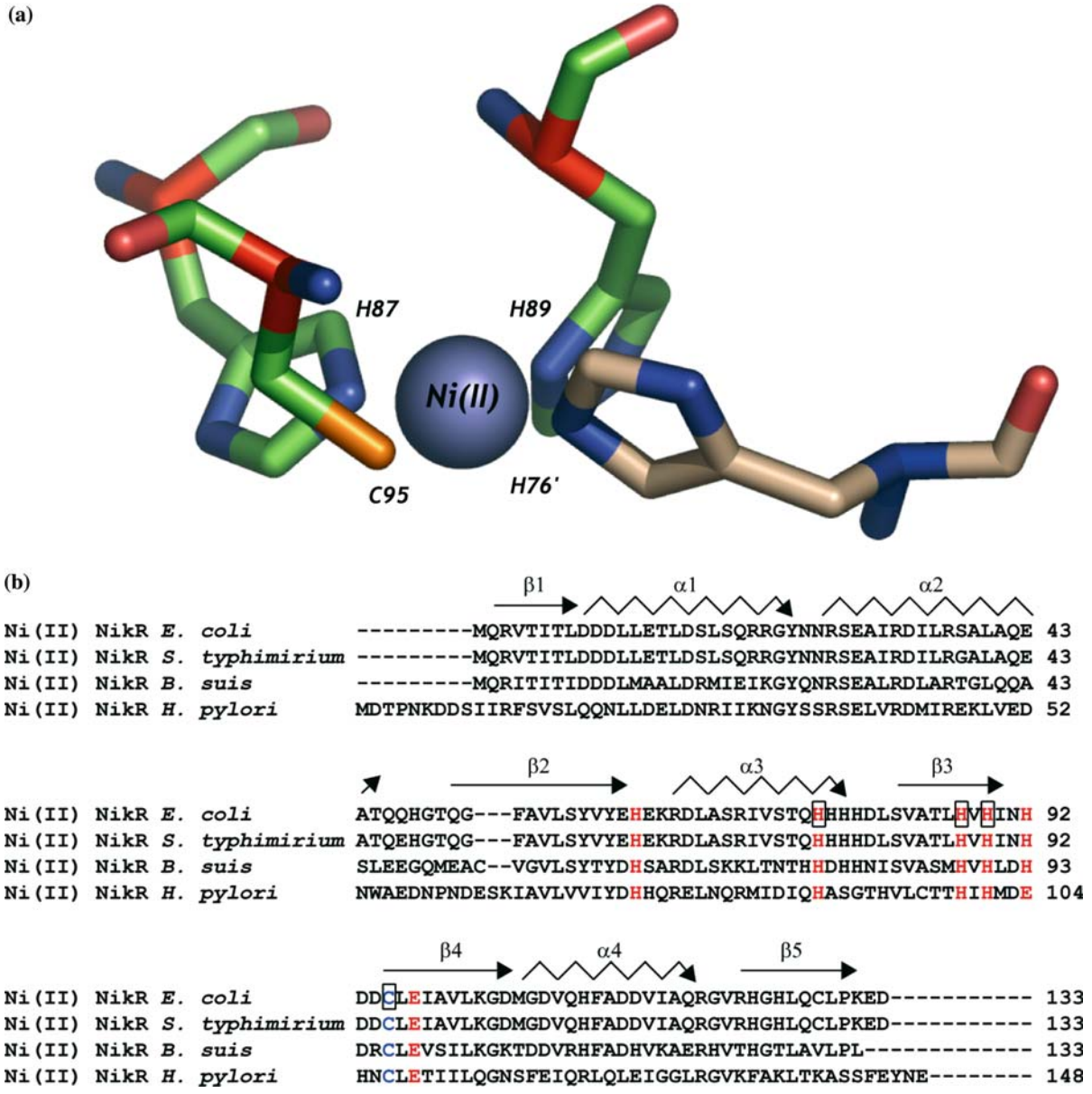


Figure 5. NikR family metalloregulators. (a) Close-up of the coordination chelate of *E. coli* NikR C-terminal regulatory domain. The nickel ion shown is colored *slate*. Three nickel ligating residues derived from one protomer (H87, H89, and C95), and H76′ from the other protomer coordinate Ni(II) in a square planar geometry. (b) Multiple sequence alignment of *E. coli* NikR, *S. typhimirium* NikR, *B. suis* NikR, and *H. pylori* NikR using Clustal W (http://www.ebi.ac.uk/clustalw) (Thompson *et al.* 1997). Ni(II) binding residues in free NikR are boxed, while residues thought to be involved in Ni(II) binding upon DNA binding are colored *red* (see text for details). Secondary structure elements are based upon the crystal structure of apo-NikR (Schreiter *et al.* 2003).

protomers within the homo-oligomer, in this case, three ligands (His 87, His89, and Cys95) from one subunit, and a fourth (His76′) from the adjacent subunit (see Figure 5a).

Surprisingly, the coordination geometry of square planar Ni(II) site becomes six-coordinate upon binding to operator-containing DNA to form a metal site composed exclusively of six N/O donors (a mixture of imidazole, carboxylate and/or solvent ligands). The structure of this DNA-bound chelate is unknown, but likely incorporates at least a subset of the original square planar donors, with the exception of Cys95, which is clearly expelled from the metal chelate (Carrington *et al.*

2003). Although the mechanism of Ni(II)-mediated allosteric positive regulation of DNA binding by NikR is not yet known, this change in Ni(II) coordination number and ligand type might suggest that Ni(II) binding induces a quaternary structural conformational change that alters the relative orientation of the N-terminal DNA binding domains relative to the core tetramerization domain (Schreiter *et al.* 2003).

Like other metal sensor protein families discussed here, the Ni(II) coordination geometries of NikR likely play a key role in defining NikR biological metal specificity. The novel N_3S square planar coordination complex in free NikR is inherently selective for Ni(II) since other transition metal ions do not easily adopt this geometry. However, recent work suggests that allosteric activation of NikR-DNA binding is determined not by N_3S square planar site exclusively, but more by occupancy of the lower affinity, octahedral site(s) where Ni(II) appears uniquely effective (Bloom & Zamble 2004). The characterization of additional NikR family members is now underway, including NikR from *B. suis*, a bacterial parasite that invades mammalian macrophages, and NikR from *H. pylori*, a human gastric pathogen (Jubier-Maurin *et al.* 2001; van Vliet *et al.* 2002, 2004). They appear to have additional regulatory functions beside nickel transport, including regulation of the expression of genes important for the survival of the organism (van Vliet *et al.* 2004). A multiple sequence alignment of NikR homologs reveal that known metal and putative metal binding ligands are strictly conserved however, suggesting that the exquisite specificity of NikR for Ni(II) will be a property shared by all NikR proteins (Figure 5b).

Conclusions

In metal sensor proteins, metal ion coordination geometry dictated by coordination number, rather than specific ligand type, is the strongest determinant for metal selectivity among closely related orthologs within the same metal sensor family, with trends largely consistent with simple expectations from fundamental inorganic chemistry. One additional general characteristic of these metal sensing sites worth pointing out is that the thermodynamic stabilities of these sites are typically extremely high (pM and fM metal ion equilibrium dissociation constants for NikR, SmtB, and ZntR/Zur, respectively, and zM for CueR) (VanZile *et al.* 2000; Outten & O'Halloran 2001; Changela *et al.* 2003). This makes it unlikely that the metal ion will move from donor to acceptor, i.e., to or from the metal sensor protein to other proteins, metallothioneins or small molecules in the cell, e.g., reduced glutathione, via the solvent. Instead, the metal might be transferred directly from donor to acceptor in a process characterized by an equilibrium constant closer to unity that might be kinetically facilitated by transient docking and rapid metal ligand exchange, like that envisioned to occur with Cu-metallochaperones (for a review, see Huffman & O'Halloran 2001). This would enable cells to maintain metal homeostasis when challenged by toxic concentrations of metal ions in the absence of large thermodynamic gradients of 'free' metal inside the cell or within other intracellular compartments. This promises to be an active area of investigation in the future.

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