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Understanding How Cells Allocate Metals Using Metal Sensors and Metallochaperones

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ABSTRACT

Each metalloprotein must somehow acquire the correct metal. We review the insights into metal specificity in cells provided by studies of ArsR−SmtB DNA binding, metal-responsive transcriptional repressors, and a bacterial copper chaperone. Cyanobacteria are the one bacterial group that have known enzymatic demand for cytoplasmic copper import. The copper chaperone and ATPases that supply cyanobacterial plastocyanin and cytochrome oxidase are reviewed, along with related ATPases for cobalt and zinc. These studies highlight the contributions of protein−protein interactions to metal speciation. Metal sensors and metallochaperones, along with metal transporters and metal-storage proteins, act in concert not only to supply the correct metals but also to withhold the wrong ones.

Introduction

A naïve expectation was that each protein would pluck the correct element from mixed-metal cell solutions and exclude all others solely based on its metal affinities. However, the full suite of protein−ligand chemistries and coordination geometries is inadequate for such perfect metal partitioning in one step. When cells are viewed as a system, they discern and account for metal ions. They acquire more of those ions that are deficient while exporting or sequestering those that are surplus. Some metals, such as mercury, are not required at all and are excluded to avoid erroneous protein associations outcompeting the correct ions. Cells manage metal−protein speciation.

Metal-sensing transcriptional regulators are useful for exploring metal selectivity *in vivo*. First, metal binding can be followed inside a cell because this triggers gene expression. Second, differences between sensor metal sites are likely to reflect selectivity. In other metalloproteins, metal sites are often constrained by diversity in catalysis (e.g., redox potential of copper), required structural folds (e.g., zinc fingers), or unrelated evolutionary histories. In a family of sensors that have diverged from a common ancestor solely to discern metals, these other factors contribute less. There is interest in understanding how cells sense metals per se, but these sensors also have utility as tools to interrogate the factors that assign metals to proteins. Here, we consider the ArsR−SmtB family but acknowledge the existence of other families of bacterial metal sensors that have been extensively studied mostly by other scientists. These include the MerR family (CueR, ZntR, PbrR, and CoaR), Fur family (Zur and Mur), DtxR, IdeR, MntR, and NikR.

ArsR−SmtB Metal Sensors

Affinity, Access, and Allostery. In 1993, we cloned and characterized a zinc sensor, SmtB, from *Synechococcus* PCC 7942.[1] The human zinc sensor MTF was cloned in the same year,[2] then the yeast zinc sensor Zap1,[3] and additional bacterial zinc sensors including ZntR and Zur.[4,5] SmtB binds to the bacterial metallothionein (SmtA) gene promoter as an apohomodimer,[6] bending the DNA by 32° (our unpublished observations) to inhibit *smtA* transcription. Zinc SmtB has a weaker affinity for DNA; hence, its occupancy of the *smt* promoter is less when zinc exceeds some critical threshold.[7,8] When the derepressed *smtA* gene is transcribed, SmtA accumulates to sequester surplus zinc.[9,10] ArsR similarly regulates the *ars* operon, which encodes an arsenical-translocating ATPase,[11] while CadC[12] was subsequently shown to regulate *cadA*, which encodes a cadmium efflux ATPase.[13] Thus, by the mid 1990s, a family of related metal sensors were known that each alleviated transcriptional repression in response to different metals or metalloids, zinc (SmtB), arsenite (ArsR), and cadmium (CadC). In the ensuing decade, additional family members were added to the ensemble including CzrA (cobalt and zinc),[14,15] NmtR (nickel and cobalt),[16] and CmtR (cadmium and lead).[17] How do these transcription factors detect and discriminate between metals?

SmtB was the first metal sensor to be structurally characterized, revealing a winged helix−turn−helix repressor with two predicted pairs of metal-binding sites per dimer (Figure 1).[6,15] Helices α3 and α4 are predicted to form the DNA-associating helix−turn−helix. *In vitro* and *in vivo* studies have confirmed the existence of one pair of metal sites associated with antiparallel carboxyl-terminal α5 helices. A second pair involves the α3 helix of the helix−turn−helix region.[18−20] The latter site recruits two further ligands from the amino-terminal region (N) of the opposing monomer (α3N). Site-directed mutagenesis revealed that α5 ligands were required for inducer recognition. The α5 mutants retained DNA binding and repression but failed to sense zinc. Ligands at α3 were not obligatory for inducer recognition *in vivo*, and these mutants behaved like wild-type SmtB.[20] Conversely, in ArsR, ligands at α3 were obligatory for inducer recogni-

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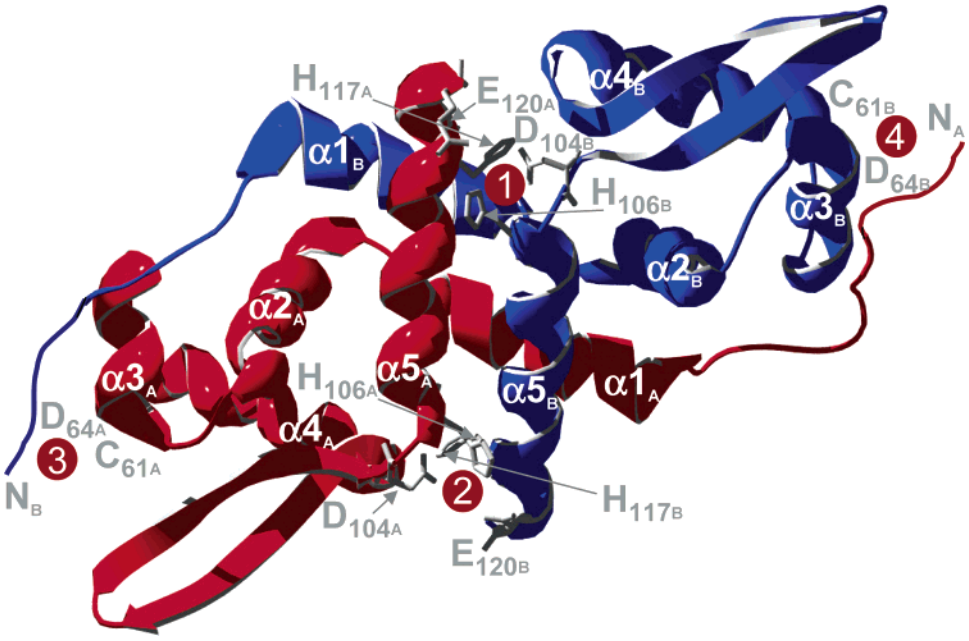

FIGURE 1. Representation of the winged-helix structure of SmtB dimers with helices α3 and α4, forming the DNA-binding regions.[6,15] The amino terminal, ca. 20 amino acids of each monomer were invisible in electron-density maps. Four metal-binding sites are located at dimer interfaces. The α5 allosteric sites (1 and 2) each include Asp_{104}, His_{106}, plus His_{117} and Glu_{120}. Sites 3 and 4 involving Cys_{61} at helix α3 plus ligands from the opposing amino-terminal (N) region are not obligatory for SmtB metal sensing.

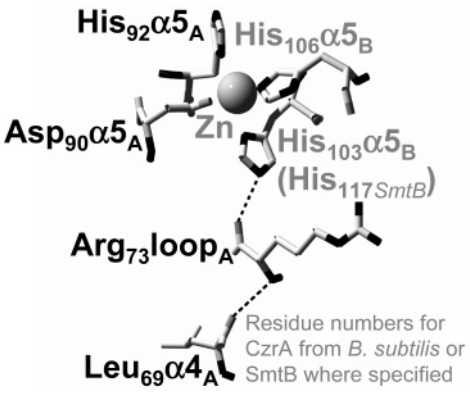

FIGURE 2. Hydrogen-bond network connects the nonliganding imidazole nitrogen of a metal-bound His of the α5 site of CzrA (or SmtB) to the DNA-associating α4 helix.[15]

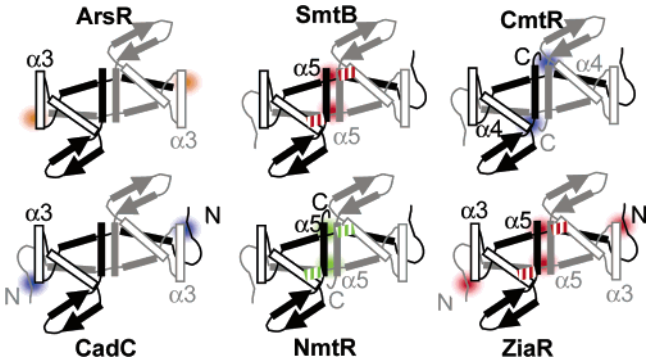

FIGURE 3. Representation of the alternative sensory sites, for oxyanions of arsenite and antimonite at α3 in ArsR (orange), zinc at α5 in dimeric SmtB and α5 plus α3 in ZiaR (red), cobalt and nickel at α5C in NmtR (green), and cadmium and lead (blue) at α3N in CadC and α4C in CmtR. β strands are indicated by arrows and α helices are indicated by boxes. Hydrogen bonds (colored bars) connect a zinc-SmtB α5 ligand to helix α4, thereby repositioning the DNA-associating helices α3 and α4 (open boxes).

tion.[21,22] It is easy to envisage metal binding at the helix−turn−helix, distorting the DNA-binding region and hence reducing the stability of ArsR−DNA complexes, but how does metal binding at more remote α5 helices modulate SmtB DNA binding? David Giedroc and co-workers uncovered a hydrogen-bond network that is established when zinc binds at the α5 site of SmtB or the related protein, CzrA (Figure 2). This connects the nonliganding imidazole nitrogen of zinc-His_{117} (SmtB numbering) to helix α4 of the opposing monomers' helix−turn−helix, repositioning these regions to become suboptimal for DNA binding.[15] The diverse ArsR−SmtB metal-sensory sites have been described as a "theme and variations model";[23] indeed, we now know of variations on at least three themes (Figure 3): (i) α3 with or without amino terminal residues "N", (ii) α5 with or without additional carboxyl-terminal residues "C", as in NmtR (below), and (iii) α4 in which the hydrogen-bond network connecting the metal-binding site of SmtB to helix α4 is replaced by direct metal-binding at α4, as in CmtR. In the zinc sensor, ZiaR, both α5 and α3N are obligatory for inducer recognition.[24]

SmtB versus NmtR. To determine how related family members detect different metals, we made pairwise comparisons of sensors with different specificities. NmtR represses transcription from the *Mycobacterium tuberculosis nmt* operator promoter, and repression is alleviated by nickel and cobalt but not zinc or any other metal tested *in vivo*.[16] This is opposite to SmtB from the cyanobacterium *Synechococcus* PCC 7942, which senses zinc but not nickel or cobalt *in vivo*. SmtB and NmtR were studied in the cytosol of a cyanobacterium and mycobacterium,

respectively. Are the differences inherent properties of the sensors or the cell types? We reconstructed NmtR metalloregulation of a reporter gene in the cyanobacterium to discover that NmtR repression of the *nmt* operator promoter was only alleviated by cobalt and not nickel, even though nickel had been the most potent elicitor in mycobacteria.[16] When cells were exposed to elevated nickel, rising to maximum permissive concentrations, the nickel content of mycobacterial cells rose from 2×10^4 to 7.4×10^5. Cyanobacterial cells only increased from 2×10^4 to 7×10^4 atoms per cell.[16] NmtR may not respond to nickel in the cyanobacterium because this element does not accumulate to high levels. This exemplifies the cytosol controlling metal access to determine which metals occupy metalloproteins *in vivo*.

In the cyanobacterial cytosol, NmtR sensed cobalt and not zinc and SmtB sensed the converse. What is the basis of this difference in selectivity that presumably must reside within the proteins themselves? Our simplistic assumption was that NmtR would have a stronger affinity for cobalt and a weaker affinity for zinc, while SmtB would show the reverse. However, NmtR has an affinity for zinc 6 orders of magnitude tighter than its affinity for cobalt, and the non-cobalt sensing SmtB has an affinity for cobalt about 2 orders of magnitude tighter than that of NmtR for cobalt. *In vitro* DNA-binding assays showed that cobalt caused dissociation of NmtR−DNA complexes at concentrations that zinc did not. Thus, even though zinc binds to NmtR more avidly than cobalt, zinc fails to impair DNA binding.[16] Site-directed mutagenesis established that NmtR has α5 metal-sensing sites, analogous to SmtB, but supplemented with two extra ligands from a short carboxyl-terminal extension. This replaces allosterically active tetradentate sites of SmtB with hexadentate sites in NmtR.[16] The extinction coefficients for *d*−*d* transitions in the 500−600 nm region for cobalt NmtR and SmtB (and SmtB mutants in α3) imply octahedral and tetrahedral coordination geometries at the respective sensory sites, confirmed by EXAFS.[16,25,26] However, Zn NmtR shows tetrahedral coordination geometry. Thus, although zinc binds to NmtR with high affinity, it does not trigger the allosteric mechanism because it binds tetrahedrally, using only a subset of the six ligands, and this protein requires hexadentate binding for allostery. These two sensors discriminate between metals by demanding different coordination geometries at the α5 helices, "*not*" to mediate the correct selective metal binding and metal partitioning, but instead to confine allosteric switching to the correct element.

NmtR versus CmtR. CmtR is another ArsR−SmtB family member from mycobacteria, but its sensitivities are perfectly inverted in relation to NmtR; CmtR senses cadmium and lead but not nickel and cobalt, while NmtR is insensitive to cadmium and lead.[17] NmtR has a tighter affinity for nickel than CmtR, while cadmium partitions to CmtR rather than NmtR. The latter was established by monitoring sulfur−metal charge-transfer bands, showing that in the presence of 0.9 equiv of cadmium the metal associated wholly with CmtR and not a competing equivalent of thiol-free NmtR. In contrast, cobalt partitions equally between the two sensors, implying closely matched affinities for cobalt.[17] Access to cobalt must either be restricted to NmtR *in vivo*, or alternatively, the allosteric mechanism in CmtR must distinguish bound cadmium versus cobalt.

Metal selectivity by metal sensors is paramount. The fidelity of their discrimination controls the abundance of different metals within cells with consequences for metal occupancy of other metalloproteins. However, even in metal sensors, selectivity does not strictly follow metal-binding preferences. Metals can partition incorrectly *in vitro*, and the wrong metals bind more tightly than the correct ones. In some cases, the wrong metals might use only a subset of ligands or a different geometry. When such differences are discerned, inappropriate triggering of the allosteric mechanism can sometimes be avoided. However, if such erroneous metalation was widespread *in vivo*, protein inactivation might become problematic. Cells also restrict access to metal-binding sites by channeling elements away from the wrong proteins. This is considered in relation to a copper metallochaperone.

Copper Metallochaperone in Copper-Importing Bacteria

Destinations for Copper in Bacteria. In eukaryotic cells, metallochaperones assist in the supply of copper to its three cytoplasmic destinations, the trans-Golgi network, superoxide dismutase, and mitochondrial cytochrome *c* oxidase (Figure 4A).[27−33] Remarkably, for most bacteria, there is currently no documented metabolic requirement for copper to cross the plasma membrane and enter the cytoplasm. *Escherichia coli* does have copper-requiring proteins in the periplasm and embedded in the plasma membrane. These include amine oxidase, superoxide dismutase, and cytochrome (quinol) oxidase. A two-component sensory system, CusR/S, detects surplus copper in the periplasm and modulates *cus* expression and hence outer-membrane export (Figure 4B). There is also a cytoplasmic copper detector, CueR, controlling production of a P_1-type ATPase, CopA, and multi-copper oxidase, CueO.[34] The latter binds four copper ions and is exported via the TAT pathway for prefolded proteins. Does CueO acquire copper in the periplasm or is it exported pre-metalated? It is possible that CueR acts to exclude all copper from the cytoplasm by regulating two proteins that both relocate stray ions to the periplasm. CueO may also oxidize periplasmic copper(I).

Gram-positive bacteria, devoid of a periplasm, do have proteins that require copper located outside the cytoplasm. For example, *Bacillus subtilis* has a caa_3-type cytochrome oxidase in which an externally located cytochrome *c* is covalently fused to subunit II of the oxidase. Copper laccase, CotA, is also found in *B. subtilis* spore coats.[35] *B. subtilis*[36] (Figure 4C) and *Enterococcus hirae*[37] (Figure 4D) have sensors to detect cytoplasmic copper, CueR and CopY, respectively, plus the CopZ copper chaperone, which interacts with the CopA copper trans-

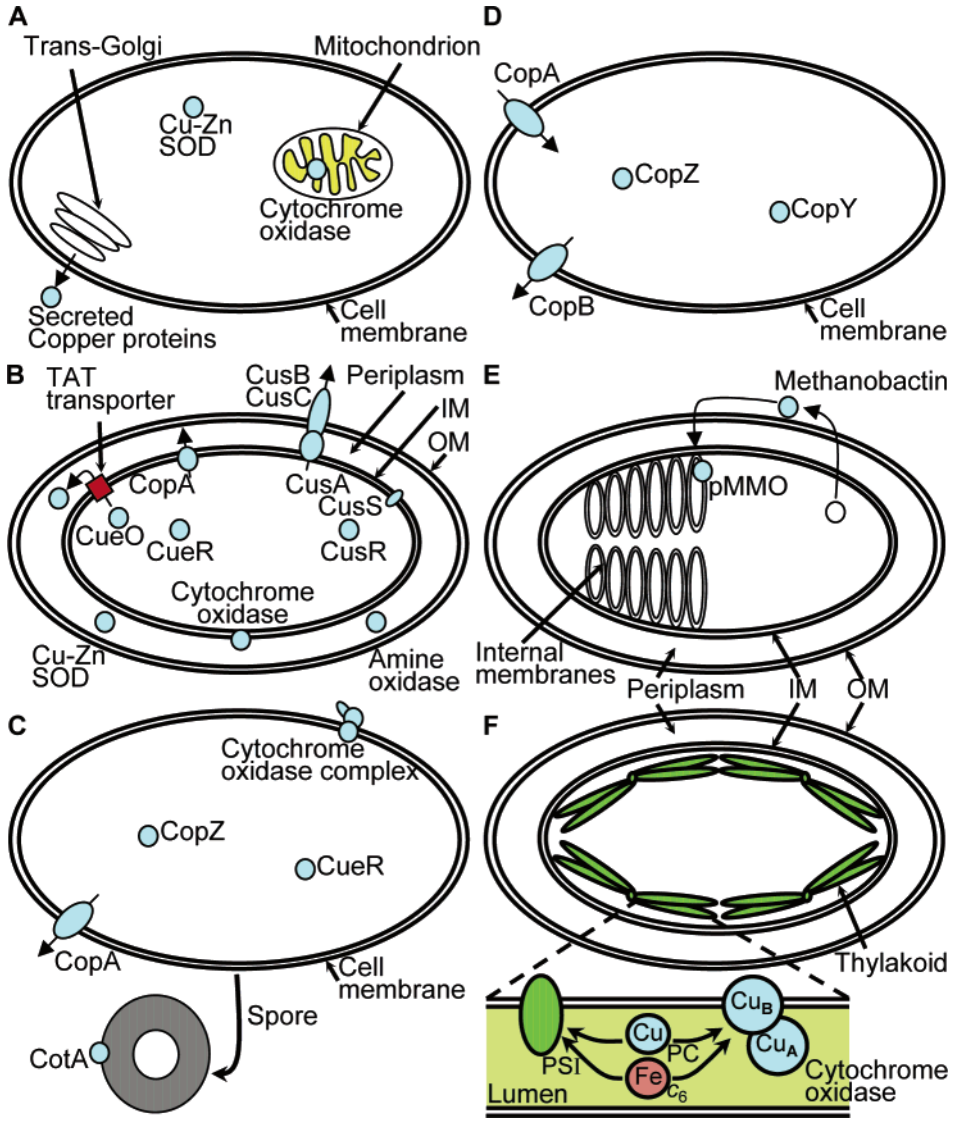

FIGURE 4. Destinations for copper: (A) in the cytoplasm of a typical eukaryote, with metal transporters and chaperones excluded for simplicity; (B) in the periplasm (IM = inner membrane, and OM = outer membrane) of *E. coli*, plus copper sensors and transporters; (C) Outside the cytoplasm of Gram positive *B. subtilis*, plus copper sensor, transporter, and chaperone; (D) Copper transporters, chaperone, and sensor of *E. hirae*; (E) Copper-requiring particulate methane monoxygenase (pMMO) within internalized membranes of methanobacteria, plus its associated copper siderophore, methanobactin; (F) Cyanobacteria contain discrete internal compartments, thylakoids, where copper is required by plastocyanin (PC) and also cytochrome oxidase. PC donates electrons to photosystem I (PSI) or cytochrome oxidase. In the absence of copper, electrons alternatively pass through heme iron in cytochrome c_6. Copper must enter the cyanobacterial cytoplasm.

porters and CopY. In *E. hirae*, CopA appears to import copper, but cytoplasmic copper enzymes are unknown in Gram-positive bacteria. Whether copper is ever routed via the cytoplasm to supply proteins located outside bacterial plasma membranes remains to be tested.

Some methane oxidizers might have an enzymatic requirement for copper to enter the cytoplasm (Figure 4E). When copper is available, they switch from using iron-requiring soluble methane monooxygenase (sMMO) to a copper-requiring particulate enzyme, pMMO. The particulate enzyme is located within internalized membranes, probably derived from the plasma membrane. The structure of pMMO[38] has not unequivocally resolved the fully active catalytic site. It may include a nonribosomally synthesized peptide-siderophore-like compound, methanobactin.[39] Alternatively, methanobactin may solely scavenge copper from the environment. pMMO might acquire copper from methanobactin within the periplasm and/or periplasmic invaginations, avoiding a requirement for copper to traverse the cytoplasm.

Many cyanobacteria contain internal membrane-bound compartments called thylakoids, which are discrete from the periplasm. Thylakoids are the site of both photosynthetic and respiratory electron transport involving the copper proteins plastocyanin and, unusually for a bacterium, cytochrome *c* oxidase (Figure 4F). The latter enzyme contains a dicopper A site oriented inside the thylakoid lumen. Plastocyanin is imported via the Sec pathway for unfolded proteins. A separate thylakoid import pathway must exist for copper. Copper-dependent change in plastocyanin production has provided invaluable insight into metalloregulation.[40] The role of thyla-

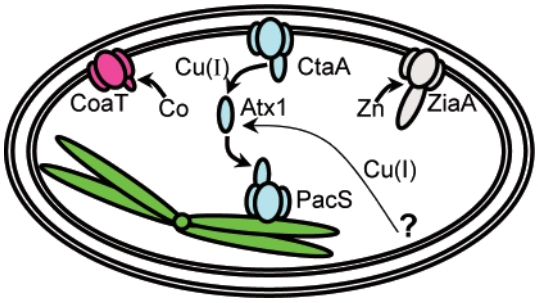

FIGURE 5. Copper trafficking to thylakoids and the multiple P-type ATPases of *Synechocystis* PCC 6803. CtaA and PacS, in conjunction with metallochaperone Atx1, deliver copper for thylakoid proteins. Atx1 partly functions without CtaA, suggesting an unknown route. ZiaA and CoaT export zinc and cobalt into the periplasm, respectively.

koidal plastocyanin in the conversion of light into chemical energy within the biosphere (especially in related plant chloroplasts) makes thylakoids one of the most important biological destinations for copper on earth. Cyanobacterial thylakoids are currently the only location for copper enzymes known to require that copper enters a bacterial cytoplasm.

Copper Trafficking to Cyanobacterial Thylakoids. Copper is trafficked to cyanobacterial thylakoids through the actions of two P-type ATPases, CtaA and PacS.[41−43] These two proteins differ from the human copper ATPases associated with Menkes' and Wilson's disease in containing only a single, rather than six, ferredoxin-like folds and metal-binding sites within amino-terminal, cytosolic domains. A metallochaperone (Atx1) interacts with these domains and is presumed to acquire copper from CtaA and donate it to PacS (Figure 5), although only the latter transfer has been tested.[44] The significance of metal donors and metal acceptors for the ATPase transport theory has been commented on elsewhere.[45] Mutants deficient in *ctaA*, *pacS*, or *atx1* have low cytochrome oxidase activity in purified membranes. They are also impaired in photosynthetic electron transport via plastocyanin as assessed spectrally *in vivo*, and their plastocyanin transcripts are less abundant. The *ctaA*·*atx1* double mutants have even lower cytochrome oxidase activity than either single mutant, implying that Atx1 can partly function without CtaA.[44] Atx1 may acquire copper from another importer.

The cyanobacterial metallochaperone copper site was analyzed by EXAFS and a solution structure resolved by NMR.[46,47] Unlike related proteins from other organisms, copper is stabilized by a third ligand derived from His_{61}-imidazole Nϵ, in addition to a pair of Cys-thiols (Figure 6). The extra nitrogen ligand from loop five may have significance for reversing the vector of copper transfer from acquisition to donation upon interaction with two different ATPases. Ivano Bertini and co-workers have analyzed interactions between copper metallochaperones from other organisms (yeast ATX1 and CopZ) and their cognate ATPases, by NMR. Loop five was displaced from the metallochaperone metal-binding sites during contact, opening the site to aid ligand invasion from the partner.[48,49] An equivalent displacement of loop five of cyano-

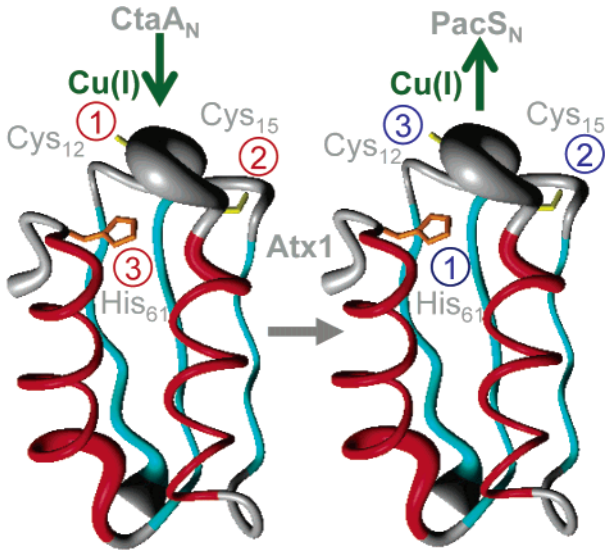

FIGURE 6. Structure of apo-Atx1. Copper is bound by imidazole nitrogen in addition to two thiols, and numbers indicate the proposed ligand sequence for copper acquisition from (red) and release to (blue) the transporters.

bacterial Atx1 solely on contact with PacS would displace His_{61} from the coordination sphere to assist copper release. Mutation of His_{61} has a different effect on two-hybrid interaction with PacS versus CtaA. It is speculated that this residue solely enters the shared site with CtaA to assist copper acquisition by Atx1.[46] Most of the mobility in apo-Atx1 is restricted to loop one containing the two cysteinyl ligands. One of these is probably the first ligand to invade the copper site of CtaA. The tumbling rate of copper Atx1 implies formation of dimers but with negligible surface contact. Experimental restraints derived from NMR analyses were satisfied by symmetrical dimers in which Cys_{12} or His_{61} but not Cys_{15} invaded the copper site of the opposing monomer.[47] On the basis of this, a most probable sequence of ligands has been proposed by which copper is passed from the cyanobacterial plasma membrane to the thylakoid and hence to plastocyanin and cytochrome oxidase (Figure 6). An all-important feature of this scheme is that copper reaches its destination without release into the cytosol, and this has pivotal significance in relation to metal selectivity.

Selectivity in Three Metal-Exporting P_1-Type ATPases. *Synechocystis* PCC 6803 has two further P_1-type ATPases, ZiaA and CoaT (Figure 5). Mutants deficient in *ziaA* are hypersensitive to zinc (not copper or cobalt);[24] mutants deficient in *coaT* are hypersensitive to cobalt (not copper or zinc); and in both cases, radioisotopic labeling implies the loss of cytoplasmic metal export.[50] Thus, these cells contain three related proteins, PacS (copper), ZiaA (zinc), and CoaT (cobalt) that select and export different metals from the cytosol. These differences could theoretically reside in the metal-regulated production of the different transporters. CoaT is made in elevated cobalt under the control of the cobalt-responsive, DNA-underwinding, transcriptional activator CoaR,[50] while ZiaA is produced in response to surplus zinc, via ZiaR derepression (Figure 7A).[24] To determine if the transporters themselves are

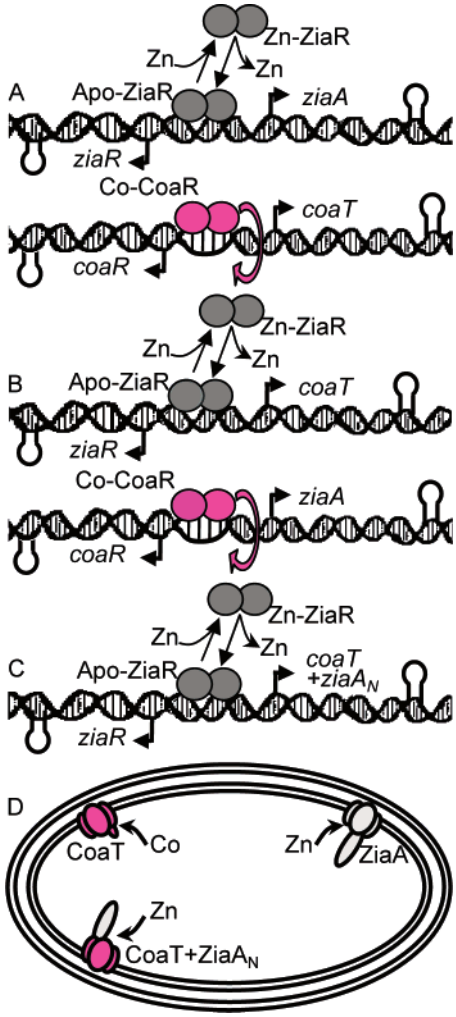

FIGURE 7. (A) ZiaR-represses *ziaA*, and this is alleviated by zinc, while cobalt CoaR activates *coaT* by a DNA-unwinding mechanism first described for mercury MerR. (B) Regulators were swapped. (C) DNA encoding the amino-terminal region of CoaT was also swapped with analogous sequences from ZiaA. (D) Zinc and not cobalt is transported by zinc-regulated CoaT if its cytosolic domain is removed and the equivalent one from ZiaA is added.

selective, we embarked upon a series of genetic manipulations.[51] First, a *zia*·*coa* double mutant was generated, which was no more sensitive to zinc than the *zia* mutant and no more sensitive to cobalt that the *coa* mutant. Into this strain, the zinc transporter was reintroduced under the control of the cobalt regulator, and separately, the cobalt transporter under the control of the zinc regulator (Figure 7B). Perhaps predictably, these constructs did not switch metal resistance conferred by the transporters. Finally, domains of the transporters were swapped, including a strain in which the amino-terminal cytosolic region of CoaT was replaced with the equivalent region of ZiaA (Figure 7C). This restored zinc resistance to the *zia*·*coa* double mutant and reduced the cell zinc content, implying that swapping the amino-terminal domain had somehow flipped selectivity of the transporter from cobalt to zinc (Figure 7D).[51] This result could be due to the loss of the amino-terminal region of CoaT, which normally prevents zinc from accessing the membrane regions of

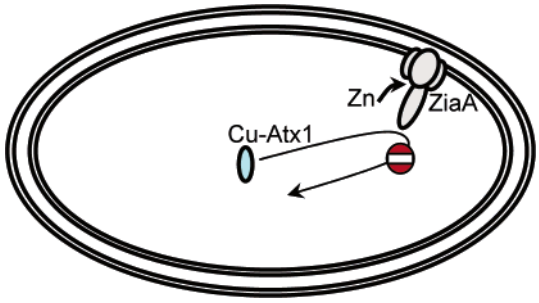

FIGURE 8. Even though ZiaA$_N$ has a higher affinity for copper than zinc, failure to interact with Atx1 can prevent aberrant access to copper *in vivo*.

CoaT, or the gain of zinc acquisition via the amino-terminal region of ZiaA. However, it establishes some role for these amino-terminal regions in metal selectivity.

Do the metal affinities of the amino-terminal regions of P_1-type ATPase match metal specificities of transport? An additional Asp in the amino-terminal metal-binding site of ZntA from *E. coli* is thought to favor zinc and cadmium relative to copper and silver. Whether this is enough to invert the order of metal affinity relative to CopA remains untested.[52] The purified amino-terminal region of ZiaA binds cobalt to give ligand−metal charge-transfer bands plus detectable *d*−*d* transitions. These features are readily bleached by zinc.[51] Thus, this domain of a transporter that selects zinc in preference to cobalt does have a greater affinity for zinc than cobalt. However, this domain also binds copper(I), but thiol−copper charge-transfer bands are not readily bleached by excess zinc. Furthermore, after competitive binding, copper(I) cochromatographs with the protein, while zinc remains unbound.[51] Metal selectivity by metal exporters, along with metal sensors, must be near the apex of the selectivity hierarchy, yet evolution has failed to achieve a binding preference at equilibrium, in favor of zinc and against copper in this domain of zinc-exporting ZiaA.

Protein Contact and Metal Access. Why does copper not preferentially bind to the amino-terminal region of ZiaA *in vivo* and interfere with its contribution to zinc homeostasis? Bacterial two-hybrid assays show that Atx1 interacts with the amino-terminal domains of the two copper transporters (PacS and CtaA) but not the analogous regions of CoaT or ZiaA,[44] not even the subdomain of ZiaA predicted to form a similar fold (Figure 8).[51] Although the metal-binding site of the amino-terminal region of ZiaA has a higher affinity for copper(I) than zinc, surface features repel Atx1 such that the site may never gain access to copper(I). It remains to be tested whether this is due to the surface charge from Glu_{13} near the copper site of Atx1 being complementary to Arg_{11} and Lys_{14} in PacS and CtaA but repulsive to Asp_{16} in ZiaA (Figure 9).[47]

Perspective and Prospective

The sensitivity of the *E. coli* copper sensor CueR (Figure 4B) is 10^{-21} M, zeptomolar. One atom per cell volume is formally nanomolar.[53] CueR is so sensitive that all free cytosolic copper atoms will be expelled through the

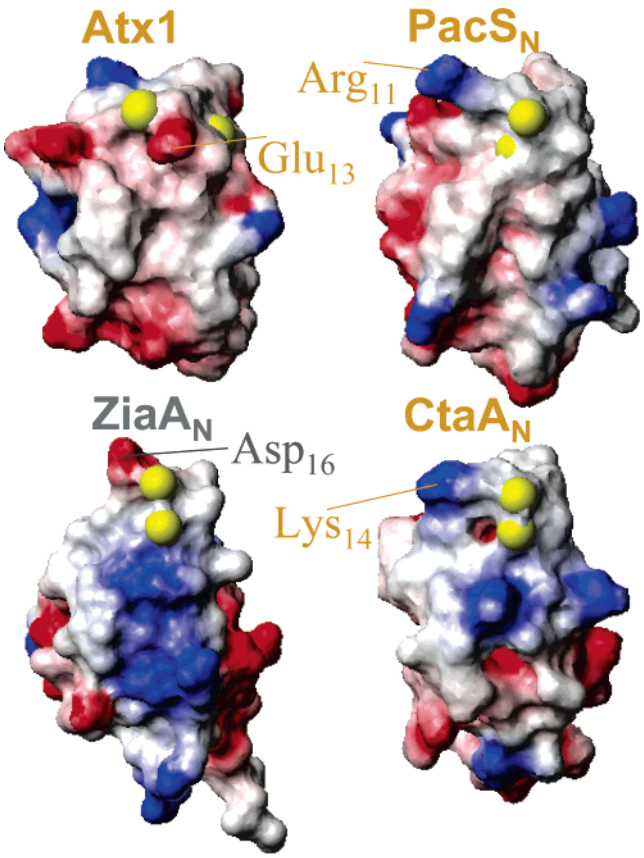

FIGURE 9. Electrostatic surfaces of Atx1[47] and of the modeled, analogously folded, soluble amino-terminal domains of the two interacting copper transporters (PacS$_N$ and CtaA$_N$) and the non-interacting zinc transporter (ZiaA$_N$).

plasma membrane of *E. coli*. This is certainly reasonable if there is no cytosolic copper demand. The requirement for the CCS copper metallochaperone to efficiently metalate Cu/Zn superoxide dismutase, despite the subfemtomolar copper affinity of the enzyme, also suggests that there is no freely available cytosolic copper in some eukaryotic cells.[54] The Irving–Williams series implied long ago that copper is highly competitive for nitrogen- and sulfur-containing protein metal-binding sites.[55] It is anticipated that copper is passed to destinations by sequential ligand exchange (Figures 5 and 6) to restrict it from sites that should be occupied by other elements but that have not evolved or may not evolve a greater affinity for the correct metal than for copper (Figure 8). Importantly, this implies that the specificity of protein–protein contact surfaces can over-ride inherent metal-binding preferences to dictate metal selectivity *in vivo*.[44,47,51]

O'Halloran and co-workers have also determined that the set point for the *E. coli* zinc sensors ZntR and Zur is femtomolar, again equating to less than one free atom per cell volume. This hints that zinc might also not be released freely into the cytosol.[56] Perhaps access to zinc must be withheld, for example by SmtA,[9,10,57–59] from sites that should be occupied by even less competitive metals such as non-heme iron. The extent of cellular metal delivery by sequential ligand exchange needs to be explored. The possibility of direct associations between apoproteins and metal importers should also be investigated.

Table 1

protein definitions	
ArsR	arsenic-sensing transcriptional repressor
SmtB	zinc-sensing transcriptional repressor (*Synechococcus*)
ZntR	zinc-sensing transcriptional activator
Zur	zinc-requiring transcriptional repressor
SmtA	bacterial metallothionein (*Synechococcus*)
CadC	cadmium-sensing transcriptional repressor
CzrA	cobalt/zinc-sensing transcriptional repressor
NmtR	nickel/cobalt-sensing transcriptional repressor (mycobacteria)
CmtR	cadmium/lead-sensing transcriptional repressor (mycobacteria)
ZiaR	zinc-sensing transcriptional repressor (*Synechocystis*)
CtaA	copper importer (*Synechocystis*)
PacS	copper exporter into the thylakoid (*Synechocystis*)
Atx1	copper metallochaperone (*Synechocystis*)
ZiaA	zinc exporter (*Synechocystis*)
CoaT	cobalt exporter (*Synechocystis*)
CoaR	cobalt-sensing transcriptional activator (*Synechocystis*)
CueR	copper-sensing transcriptional activator

A consequence of cells containing binding sites with higher affinity for the wrong metal than the correct metal, for example, copper versus zinc at the amino terminus of ZiaA, is a potential for metals to associate with high affinity but aberrant sites *in vivo*, so-called thermodynamic traps.[34] Do metals such as copper and zinc commonly mislocate to aberrant sites in response to metal excess, following liberation after oxidative stress or because of an accumulation of erroneous trafficking events in aging cells? Proteomic methods to monitor metal–protein associations are needed. To what extent does metal mislocation to traps underlie links between these elements and several common disorders including some neurological diseases that impair the quality of life in old-age? [33,60,61]

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