Investigating the Role of Zinc and Copper Binding Motifs of Trafficking Sites in the Cyanobacterium *Synechocystis* PCC 6803

Adriana Badarau, Arnaud Baslé, Susan J. Firbank, and Christopher Dennison*

Institute for Cell and Molecular Biosciences, Medical School, Newcastle University, Newcastle upon Tyne, NE2 4HH, United Kingdom

Supporting Information

**ABSTRACT:** Although zinc and copper are required by proteins with very different functions, these metals can be delivered to cellular locations by homologous metal transporters within the same organism, as demonstrated by the cyanobacterial (*Synechocystis* PCC 6803) zinc exporter ZiaA and thylakoidal copper importer PacS. The N-terminal metal-binding domains of these transporters (ZiaAN and PacSNN, respectively) have related ferredoxin folds also found in the metallochaperone Atx1, which delivers copper to PacSNN, but differ in the residues found in their M/IXCXXC metal-binding motifs. To investigate the role of the nonconserved residues in this region on metal binding, the sequence from ZiaAN has been introduced into Atx1N and PacSNN, and the motifs of Atx1N and PacSNN swapped. The motif sequence can tune Cu(I) affinity only approximately 3-fold. However, the introduction of the ZiaAN motif (MDCTSC) dramatically increases the Zn(II) affinity of both Atx1N and PacSNN by up to 2 orders of magnitude. The Atx1N mutant with the ZiaAN motif crystallizes as a side-to-side homodimer very similar to that found for [Cu(I)2BCA2] (Badarau et al. *Biochemistry* 2010, 49, 7798). In a crystal structure of the PacSNN mutant possessing the ZiaAN motif (PacSNN ZiaAN), the Asp residue from the metal-binding motif coordinates Zn(II). This demonstrates that the increased Zn(II) affinity of this variant and the high Zn(II) affinity of ZiaAN are due to the ability of the carboxylate to ligate this metal ion. Comparison of the Zn(II) sites in PacSNN ZiaAN structures provides additional insight into Zn(II) trafficking in cyanobacteria.

Zinc and copper are essential metal ions for most organisms but exhibit a number of important differences. The biological functions of copper primarily utilize its redox activity, which contributes to the toxicity of this metal. Redox-inactive zinc is more abundant in biological systems and is required by many more proteins. The tight binding of zinc and copper to biological metal sites means that the intracellular availability of both has to be carefully controlled. Copper trafficking generally involves metallochaperones that deliver the metal to specific targets by ligand-exchange reactions. No zinc metallochaperone is currently known, although metallothioineins involved in zinc storage have been proposed to also act as a direct source of zinc for target proteins.

Zinc export from the cytosol of the cyanobacterium *Synechocystis* PCC 6803 occurs via the P-type ATPase ZiaA. In the same organism, the P-type ATPase PacS, along with the copper metallochaperone Atx1, traffic copper to the thylakoids for photosynthesis and respiration. ZiaAN and PacSNN each possess a single N-terminal metal-binding domain (MBD; ZiaAN and PacSNN, respectively) structurally similar to Atx1 in having a M/IXCXXC metal-binding motif anchored on a ferredoxin (βαββαβ) fold (Figure 1). ZiaAN is unusual in having an unstructured C-terminal extension that contains seven His residues that are involved in Zn(II) binding. We have recently shown that the Zn(II) affinity of ZiaAN is up to 2 orders of magnitude higher than those of PacSNN and Atx1. The Cu(I) affinities of copper and zinc trafficking proteins in *Synechocystis* all fall within approximately 1 order of magnitude at pH 7.0. However, the Cu(I) affinity of Synechocystis Atx1 is almost 10-fold greater than that of PacSNN (and ZiaAN), and this Atx1 can dimerize in the presence of Cu(I), which enhances its Cu(I) affinity. The Cu(I) affinities of the trafficking sites are at least 6 orders of magnitude greater than their Zn(II) affinities, consistent with theoretical studies, yet Atx1 has been proposed to be able to bind zinc in vivo. The non-Cys residues in the M/IXCXXC motifs of metal-trafficking proteins have been implicated in metal binding and transfer. This includes contributing to Zn(II) coordination in a Zn(II)-transporting ATPase form *Escherichia coli* (*E. coli*), facilitating the rate and extent of dissociation of the Atx1–Cu(I)–BCA complex [BCA (bicinchoninic acid) is a tight Cu(I) ligand used as a copper-transfer partner mimic], influencing the flexibility of this part of the protein, and forming potentially important hydrogen-bonds, particularly with the Cys ligands. In this work, we use the *Synechocystis* system to help understand the importance of the non-Cys residues in M/IXCXXC motifs on the binding of zinc and copper. We have grafted the motif from ZiaAN onto both Atx1 (Atx1 ZiaAN) and PacSNN (PacSNN ZiaAN) and have swapped these regions between Atx1 and PacSNN, giving Atx1 PacSNN and PacSNN Atx1, respectively (Figure 1C). These mutations have a limited effect (maximum ∼3-fold) on Cu(I) affinity. However, introducing the ZiaAN loop has a dramatic influence on the

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Zn(II) affinities of both Atx1 and PacSN. In its crystal structure, Zn(II)−Atx1ZiaAN forms a side-to-side dimer with the monomers bridged by a single Zn(II) ion. The introduced Asp11 residue on the MDC12TSC15 motif is involved in an intermolecular hydrogen bond with Ser14 from the adjacent molecule. In a crystal structure of Zn(II)−PacSNZiaAtx1, the metal ion is coordinated by the carbonylate of the corresponding Asp residue, which must be the cause of the enhanced Zn(II) affinity. The comparison of Zn(II)−PacSNZiaAtx1 crystal structures provide additional insight into potential intermediate sites formed during Zn(II) trafficking in Synechocystis.

■ MATERIALS AND METHODS

Site-Directed Mutagenesis. The Atx1ZiaAtx, Atx1PacSN, PacSNZiaAtx, and PacSNAtx variants (Figure 1C) were generated using QuickChange mutagenesis (Stratagene) with pETATX1 (encoding full length Atx1)10 and pETPACS71 (encoding PacSN) as templates and the primers given in Table S1 in the Supporting Information. Both strands of all DNA constructs were purified, reduced, and quantified as previously reported,12,14,23 and the Atx1ZiaAtx, Atx1PacSN, PacSNZiaAtx, and PacSNAtx variants were verified by mass spectrometry. Far-UV (185−250 nm) circular dichroism (CD) spectra14 and analytical gel filtration chromatography12 were performed as described previously. The dimerization constant (Kdimer) for Cu(I)−Atx1PacSN was determined by gel filtration as described previously.12

Zinc Titrations and the Determination of Zn(II) and Cu(I) Affinities. Titrations of Zn(II) into apo-proteins were performed in 25 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes) pH 7.4 plus 100 mM NaCl and monitored for changes in absorbance at 240 nm on a λ35 UV/vis spectrophotometer (Perkin-Elmer).14 Zn(II) affinities were measured using the competitive chelator RhodZin-3 in 25 mM Hepes pH 7.4 plus 100 mM NaCl. Data were fit to a model (eq 1) considering a single species (ZnP, where P is the apo-protein) for Atx1ZiaAtx and PacSNZiaAtx to obtain the Zn(II) affinity of the apo-protein (KZn) and two species (ZnP and ZnP2, see eq 2) for His61Tyr Atx1, Atx1PacSN, and PacSNAtx to determine both KZn and the affinity of the apo-protein for zinc-protein (KCu). Below are eqs 1 and 2:

\[
P = \left( \frac{[Zn] - [ZnL]}{[L]} \right) \left( K_{Cu} \frac{[L]}{[Cu]} + K_{Zn} \frac{[ZnL]}{[L]} \right)
\]

(1)

\[
P = A \left( 1 + \frac{[ZnL]}{[L]} \right) K_{Zn} + 2 \frac{[ZnL]}{[L]} \frac{K_{Cu}}{[Cu]} A^2
\]

(2)

where

\[
A = \frac{2 \frac{[ZnL]}{[L]} \frac{K_{Cu}}{[Cu]} K_{Zn}}{[L] K_{Cu} + 4 ([ZnL] - [Zn]) \frac{K_{Cu}}{[Cu]} K_{Zn}}
\]

and where [L], [P] and [Zn] represent total RhodZin-3, protein, and zinc concentrations, respectively, and KCu is the zinc affinity of RhodZin-3.

For Cu(I) affinity (KCu) determinations, the chromophoric ligand bathocuproine disulfonate (BCS) was used in 20 mM Hepes pH 7.0 plus 200 mM NaCl. Data were fit to a 1:1 Cu(I):protein model (eqs 4 and 5),14,23 except in the case of Atx1PacSN, for which data at multiple Cu(I) concentrations were fit to a model that also considers dimerization of the Cu(I)−protein (eq 6)14 using a Kdim of 5.7 × 104 M−1 (vide infra). Below are eqs 4−6:

\[
[L] = 2 [Cu_{L2}] + \frac{[Cu] + [Cu_{L2}] [Cu_{L2}]}{[Cu] + [Cu_{L2}] [Cu_{L2}]}
\]

(4)

\[
[P] = \left( \frac{[Cu] - [Cu_{L2}]}{[Cu] - [Cu_{L2}]} \right) [L] - 2 [Cu_{L2}] \frac{[Cu]}{[Cu] - [Cu_{L2}]}
\]

(5)

\[
[P] = [Cu] - [Cu_{L2}] + \beta \left( \frac{[L]}{[Cu] - [Cu_{L2}]} \right) \left( \frac{K_{Cu}}{[Cu] - [Cu_{L2}]} \right) K_{Cu} K_{dim}
\]

(6)

where [L], [P] and [Cu] represent total BCS, protein, and copper concentrations, respectively, and β is the formation constant of [Cu(BCS)2]2−.

Protein Crystallization, X-ray Data Collection, Structure Determination, and Refinement. Atx1ZiaAtx (25 mg/mL) loaded with 1 equiv of Zn(II) in 20 mM Hepes pH 7.0 and 100 mM NaCl was crystallized anaerobically from 1.6 M trisodium citrate pH 6.5 using the hanging drop method of vapor diffusion (1 μL protein and 0.5 mL well solution). PacSNZiaAtx (10 mg/mL) loaded with 1 equiv of Zn(II) in 20 mM Hepes pH 7.0 plus 35 mM NaCl was crystallized from 0.1 M Hepes pH 7.5 plus 10% (w/v) PEG 4000 and 5% (w/v) isopropanol (condition 1) using the sitting drop method (250 nL protein and 100 μL well solution). A second crystal form of

![Figure 1](image-url)
Zn(II)–PacSN<sub>ZiaAN</sub> was obtained from 20% (w/v) PEG 3350 plus 0.2 M NaF (condition 2). All crystals were frozen using N-paratone oil as the cryoprotectant. Diffraction data were collected at 100 K on beamline I02 at the Diamond Light Source (Didcot, U.K.). The identity of the metal was confirmed by calculation of an anomalous difference Fourier map using additional data sets collected both above [high-energy remote (hrm)] and below [low-energy remote (lrm)] the zinc K-edge.
RESULTS AND DISCUSSION

Protein Purification and Characterization. Mass spectra (Table S2 in the Supporting Information) show that Atx1ZiaAN, PacSN, and PacSN, are purified without their N-terminal Met residue. Atx1ZiaAN is isolated as a mixture of full length protein and protein missing Met1 (Table S2 in the Supporting Information). CD spectra (Figure S1 in the Supporting Information) demonstrate fully folded proteins and domains in all cases.

Oligomerization State of the Apo and Metal-Loaded Proteins. For most proteins studied, the addition of Zn(II) causes a decrease in absorbance at 240 nm (Figure 2 and Figure S2 in the Supporting Information) in the UV/vis spectrum [the absorbance at 240 nm does initially increase upon the addition of Zn(II)] to apo-PacSN, Atx1PacSN, and Atx1PacSN, are purified without their N-terminal Met residue. Atx1ZiaAN is isolated as a mixture of full length protein and protein missing Met1 (Table S2 in the Supporting Information). CD spectra (Figure S1 in the Supporting Information) demonstrate fully folded proteins and domains in all cases.

The change in absorbance at this wavelength upon Zn(II) addition does give insight into the stoichiometry of the complexes formed. The Zn(II) titrations (Figure 2 and Figure S2 in the Supporting Information) show an inflection point at ~0.5 equiv, followed by a plateau after 1 equiv, for all proteins except Atx1ZiaAN, for which the decrease in absorbance at 240 nm is linear up to 1 equiv (Figure 2A). These data indicate the formation of a single Zn(II)-form (ZnP) for Atx1ZiaAN, as previously seen for ZiaAN,14 and two Zn(II)-loaded species (ZnP and ZnP2) for PacSN, and Atx1PacSN, and Atx1PacSN, as found for wild type (WT) Atx1 and PacSN.14 Consistent with this, most of these proteins elute as dimers from a gel filtration column when loaded with 0.5 equiv of Zn(II) (Figure 3 and Figure S3 and Table S3 in the Supporting Information), whereas Atx1ZiaAN elutes as a monomer, most probably as a mixture of apo- and Zn(II)-protein (Figure 3A and Table S3 in the Supporting Information). The elution volume of PacSN, loaded with 0.5 equiv of Zn(II) decreases (apparent molecular weight increases) with increasing protein concentration, consistent with a relatively weak ZnP2 dimer (Figure 3B and Table S3 in the Supporting Information). Atx1ZiaAN and PacSN, elute as monomers when loaded with 1 equiv of Zn(II) (Figure 3A,B and Table S3 in the Supporting Information). All of the other proteins have a greater tendency to dimerize at the relatively high protein concentrations used for the gel filtration experiments (90–200 μM) and are recovered with approximately 0.5 Zn(II) equiv (Figure S3 and Table S3 in the Supporting Information).

We have previously shown that although WT apo-Atx1 is a monomer, when the protein is loaded with 1 equiv of Cu(I) [Cu(I)–Atx1, it elutes from a gel filtration column as a dimer. However, the elution volume increases upon lowering the
Table 2. Cu(I) and Zn(II) Affinities (K_{Cu} and K_{Zn} Values, Respectively) and Affinities of the Apo-Protein for the Zn(II)-Protein (K_{Zn2} Values)\textsuperscript{a}

| Protein          | Cu(I) K_{Cu} (M\textsuperscript{-1}) | Zn(II) K_{Zn} (M\textsuperscript{-1}) |
|------------------|----------------------------------|----------------------------------|
| WT Atx1\textsuperscript{b} | (4.7 ± 0.7) \times 10\textsuperscript{−17} | (7.2 ± 0.3) \times 10\textsuperscript{−10} |
| His61Tyr Atx1    | (1.8 ± 0.3) \times 10\textsuperscript{−17} | (2.8 ± 0.3) \times 10\textsuperscript{−10} |
| Atx1\textsuperscript{PacSN} | (5.6 ± 0.1) \times 10\textsuperscript{−17} | (2.5 ± 0.1) \times 10\textsuperscript{−10} |
| Atx1\textsuperscript{ZiaAN} | (1.4 ± 0.5) \times 10\textsuperscript{−17} | (2.5 ± 0.1) \times 10\textsuperscript{−10} |
| WT PacSN\textsuperscript{b} | (7.8 ± 0.7) \times 10\textsuperscript{−18} | (4.2 ± 0.4) \times 10\textsuperscript{−10} |
| PacSN\textsuperscript{Atx1} | (1.3 ± 0.2) \times 10\textsuperscript{−17} | (5.2 ± 1.0) \times 10\textsuperscript{−10} |
| PacSN\textsuperscript{ZiaAN} | (8.5 ± 1.5) \times 10\textsuperscript{−18} | (1.7 ± 0.2) \times 10\textsuperscript{−10} |
| ZiaAN\textsuperscript{b} | (6.5 ± 1.0) \times 10\textsuperscript{−18} | (1.1 ± 0.1) \times 10\textsuperscript{−10} |

\textsuperscript{a}Cu(I) affinities were determined in 20 mM Hepes pH 7.0 plus 200 mM NaCl, and Zn(II) affinities were determined in 25 mM Hepes pH 7.4 plus 100 mM NaCl. \textsuperscript{b}From ref 14. \textsuperscript{c}Values for the monomeric protein determined from titrating apo-protein into [Cu(BCS)]\textsuperscript{3−} at multiple Cu(I) concentrations.

Figure 5. Titration of [Cu(BCS)]\textsuperscript{3−} with apo-Atx1\textsuperscript{PacSN} (A) and apo-Atx1\textsuperscript{ZiaAN} (B) in 20 mM Hepes pH 7.0 plus 200 mM NaCl. In (A), the [Cu(BCS)]\textsuperscript{3−} concentration ranges from 2.0 to 16.0 μM in the presence of an excess of BCS (46–168 μM). In (B), the [Cu(BCS)]\textsuperscript{3−} concentration is 14.0 μM with an excess of BCS (72 μM) present. The fit of the data in (A) to eq 6 using a dimerization constant (K_{dim}) of 5.7 \times 10\textsuperscript{4} M\textsuperscript{−1} gives a K_{Cu} of (5.6 ± 0.1) \times 10\textsuperscript{−17} M\textsuperscript{−1}, and the fit of the data in (B) to eq 5 gives a K_{Cu} of (1.8 ± 0.1) \times 10\textsuperscript{−17} M\textsuperscript{−1}.

Protein concentration below 100 μM, indicative of dimer dissociation in this concentration range, and an equilibrium constant (K_{dim}) of (5 ± 2) \times 10\textsuperscript{5} M\textsuperscript{−1} has been determined.\textsuperscript{12} The dimerization constant (K_{dim}) of WT Atx1 in 20 mM Hepes pH 7.0 plus 200 mM NaCl, and ZiaAN have been crystallized. Atx1ZiaAN loaded with 1 equiv of Cu(I) elute as monomers on a gel-filtration column (Figure S3 and Table S3 in the Supporting Information), whose side chain hydrogen bonds with the Cys residues in the native IACEAC motif of Atx1. The largest changes in Zn(II) affinity by a similar amount as that of WT Atx1 and a His residue at this key location, whose side chain hydrogen bonds with the Cys ligands (Figure 1), favors dimerization. All of the apo-variants loaded with 1 equiv of Cu(I) elute as monomers on a gel-filtration column (Figure 3 and Figure S3 and Table S3 in the Supporting Information) except for Cu(I)−Atx1\textsuperscript{PacSN} which elutes as a dimer (Figure S3A and Table S3 in the Supporting Information). A dimerization constant of (6 ± 1) \times 10\textsuperscript{4} M\textsuperscript{−1} was determined for Cu(I)−Atx1\textsuperscript{PacSN} (Figure S4 in the Supporting Information), ~10-fold lower than that of WT Atx1.\textsuperscript{12} This lowered dimerization constant for Atx1\textsuperscript{PacSN} and the absence of dimer formation for Atx1\textsuperscript{ZiaAN} demonstrate that residues in the native IACEAC motif of Atx1 contribute to the stability of the dimer formed in the presence of Cu(I).

Zinc(II) and Copper(II) Affinities. Introducing the metal-binding motif of Atx1 into PacSN has almost no effect on Zn(II) affinity (K_{Zn}), whereas replacing the sequence of Atx1 with that of PacSN decreases the Zn(II) affinity 3-fold (Figure 4A and Table 2). The His61Tyr Atx1 mutation decreases the Zn(II) affinity by a similar amount (Table 2), and this loop 5 residue is therefore not involved in Zn(II) binding. The affinity of apo-protein for Zn(II)-protein (K_{Zn2}) is enhanced 2- to 3-fold in Atx1\textsuperscript{PacSN} and PacSN\textsuperscript{Atx1} but is almost unaltered by the His61Tyr Atx1 mutation (Table 2). The largest changes in Zn(II) affinity (up to 40-fold) result from introducing the ZiaAN sequence into PacSN and Atx1 (Figure 4B and Table 2). Atx1\textsuperscript{ZiaAN} has the highest Zn(II) affinity of all the proteins studied, ~2- and 15-fold tighter than those of ZiaAN and PacSN\textsuperscript{ZiaAN}, respectively (Table 2).

The Cu(I) affinity (K_{Cu}) of monomeric WT Atx1 is approximately an order of magnitude greater than those of WT PacSN and ZiaAN at pH 7 (Table 2).\textsuperscript{14} Some of this difference is due to the presence of His61 on loop 5 of Atx1 because replacement with a Tyr, the residue found in this position in both PacSN and ZiaAN, results in a ~2.5-fold decrease in Cu(I) affinity (Table 2).\textsuperscript{14} The introduction of the metal-binding motif of PacSN has almost no effect on Cu(I) affinity of Atx1, but K_{Cu} decreases ~2- to 3-fold in Atx1\textsuperscript{ZiaAN} (Figure 5 and Table 2). The introduction of the Atx1 loop into PacSN (in PacSN\textsuperscript{Atx1}) does increase the Cu(I) affinity ~3-fold (Table 2), whereas the Cu(I) affinity of PacSN\textsuperscript{ZiaAN} is very similar to that of PacSN (Table 2). The non-Cys residues in the loop can influence the Cu(I) affinity by a similar amount as that seen upon mutating the loop 5 residue. These mutations all change the second-coordination sphere, which has a small effect on the Cu(I) affinity of copper-trafficking proteins, with the most important contribution being from residues that can influence the pK_{a} values of the Cys ligands.\textsuperscript{23,30}

Crystal Structures of Zn(II)−Atx1\textsuperscript{ZiaAN} and Zn(II)−PacSN\textsuperscript{ZiaAN}. To gain insight into the structural causes of the large changes in Zn(II) affinity, Zn(II)−Atx1\textsuperscript{ZiaAN} and Zn(II)−PacSN\textsuperscript{ZiaAN} have been crystallized. Atx1\textsuperscript{ZiaAN} loaded with 1 equiv of Zn(II) crystallizes as a Zn(II)-bridged dimer (Figure 6), an arrangement that is probably relevant for all the ZnP$_2$ forms that we have observed in solution (Table S3 in the Supporting Information). This arrangement (contact area ~450 Å$^2$) is remarkably similar to that of the side-to-side Cu(I)−Atx1
distal from the metal site. Zn(II)−PacSN\textsuperscript{ZiaAN} is a monomer in solution (Figure 3B and Table S3 in the Supporting Information), and this crystallographic dimer is therefore an artifact. The metal site structure in this form of Zn(II)−PacSN\textsuperscript{ZiaAN} is similar to that found in the condition 1 crystal structure except that Asp13 is replaced by a water ligand with a Zn(II)−O distance of ~2.1 Å (Figure 7B). The carboxylate group of Asp13 points away from the metal site, is solvent exposed, and is not involved in any interactions.

Attempts to crystallize ZiaAN have been unsuccessful, and NMR studies could not determine the structure of the high affinity Zn(II) site.\textsuperscript{13} NMR also has been used to investigate the MBD of the related Zn(II)-exporting ATPase from E. coli (ZntA).\textsuperscript{18} In this case a (Cys)\textsubscript{2}Asp Zn(II) site has been suggested, but because of the limitations of NMR data, neither the precise Zn(II) coordination number nor the geometry of the site could be resolved. This NMR study also suggested the possibility of a water ligand completing the coordination environment because of the solvent exposure of the Zn(II) site. Surprisingly, a recent NMR study of cyclic peptides that mimic Cu(I)- and Zn(II)-binding CXCC motifs (MTCGSCSRPG and MDCGSCSRPG, respectively) has found that the Asp residue (underlined) coordinates Cu(I) but not Zn(II).\textsuperscript{39} The crystal structures of Zn(II)−PacSN\textsuperscript{ZiaAN} are the first of a Zn(II) site bound by a CXCC motif involved in zinc transport. These structures provide strong evidence that the Asp residue preceding the CXCC motif binds Zn(II) in a monodentate fashion and that a water ligand can occupy the fourth coordination position of a tetrahedral site in the MBDs of Zn(II)-transporting proteins.

Insight into Zn(II) Trafficking Provided by the M/IXCXC Motif Variants. The sequence of the metal-binding motif has a much more significant influence on Zn(II) than Cu(I) affinities, and introducing the ZiaAN sequence into Atx1 and PacSN increases the Zn(II) affinity by up to ~40-fold. This is due to the side chain of Asp13 coordinating Zn(II), as seen in a Zn(II)−PacSN\textsuperscript{ZiaAN} crystal structure (Figure 7A), which must also be the cause of the higher Zn(II) affinity of ZiaAN. The two crystal forms of PacSN\textsuperscript{ZiaAN} have Zn(II) sites with Cys,His coordination, with either the carboxylate of Asp13 or a water molecule as the fourth ligand. A carboxylate group has a lower affinity for Zn(II) than Cys and His,\textsuperscript{34} consistent with replacement of Asp13 and not the other ligands by water. The coordination of Zn(II) by Asp18 in ZiaAN tunes its Zn(II) affinity so that it is tighter than those of the Cu(I)-trafficking proteins (with two Cys ligands) but below that of the Zn(II) sensor (His\textsubscript{Asp} coordination for SmtB from the cyanobacterium Synechococcus).\textsuperscript{35,36} It has been suggested that Asp18 prevents ZiaAN from forming a stable complex with Cu(I)−Atx1.\textsuperscript{37} A negatively charged residue close to the CXCC motif appears to be conserved in ATPases for metals (divalent) other than copper, and repulsion has been suggested as a common mechanism to prevent the binding of Cu(I). The presence of an Asp adjacent to the first Cys of the CXCC motif has almost no effect on Cu(I) affinity, as it is not required for coordination but is important for Zn(II) binding. With Zn(II) bound to ZiaAN, the negative charge of Asp13 will no longer contribute to repulsing Atx1. As observed in our crystal structures, this Asp can readily dissociate, which may occur as Zn(II) is subsequently trafficked, allowing the negative charge to help prevent unwanted interactions (vide infra). The increase in Zn(II) affinity due to the introduction of an additional Zn(II) ligand appears to be sufficient to allow the MBDs of zinc and...
copper transporters that have very similar structures to discriminate between these metals. It is therefore likely that the Zn(II) affinity of ZiaAN (10^{-10} M) is in the range of physiological free zinc concentrations in *Synechocystis*, although this has not been determined. This is supported by the observation that in *E. coli* up-regulation of ZntA expression occurs at nanomolar levels of intracellular free Zn(II), which matches the Zn(II) affinities of both the MBD and the transmembrane site of ZntA (~10^{-8} M^{-1}).

The His residue (His48) involved in Zn(II) coordination in the PacS\_N\_ZiaAN structures belongs to an adjacent molecule. The recruitment of this His ligand only occurs at the high protein concentrations required for crystallization [Zn(II)–PacS\_N\_ZiaAN is monomeric in dilute solution (Figure 3B and Table S3 in the Supporting Information)] and is probably replaced by a water ligand in solution. However, in ZiaAN, His residues from the unstructured C-terminal interact in solution with Zn(II) bound to the CXXC site. These interactions were proposed to either aid metal transfer or alter intramolecular interactions. The observation that the side chain of Asp13 can be replaced by water when a His ligand is present highlights the fluxionality of this Zn(II) site, which will assist Zn(II)-trafficking. Our structures suggest two possible intermediates involving one of the His residues from the unstructured region of ZiaAN, which could be important for zinc transfer. The coordination of Zn(II) by two His residues from the C-terminal region of ZiaAN (as well as by two Cys residues) would result in loss of the Asp ligand, enabling it to maintain a repulsive interaction with Atx1, to potentially hinder Cu(I) binding.

When the CXXC site of Atx1 binds 1 equiv of Zn(II), an exposed Zn(II) site with Cys\_H\_O\_ coordination will be present, which will be susceptible to ligand exchange reactions. This form may play a role in Zn(II) trafficking as it has been suggested that Atx1 can bind zinc in *Synechocystis*. The unsaturated nature of such a Zn(II) site also makes it prone to coordinating additional ligands, such as Asp18 in ZiaAN. Two additional Cys ligands can also be recruited from a second protein molecule, as seen in the crystal structure of Zn(II)–Atx1\_ZiaAN (Figure 6), and as indicated for other proteins in this work, and also WT Atx1, by studies in solution (Figures 2 and 3, and Figures S2 and S3 and Table S3 in the Supporting Information). These tetrahedral sites are reminiscent of Zn(II) structural sites, and their buried nature suggests they have limited functionality for Zn(II) trafficking but may play a role in storing the metal. However, given the fact that these dimers are relatively weak, there is likely fast dimer-monomer exchange so that Zn(II) can be easily accessed for the supply of endogenous Zn(II)-binding proteins. We have recently shown that heterodimers between partner proteins (e.g., Atx1 and PacS\_N) are formed in the presence of Zn(II) and are more stable than the corresponding homodimers, and may have a role in regulating the activity of copper-transporting ATPases.

## CONCLUSIONS
The Zn(II) affinity of the MBD of a cyanobacterial zinc transporter is greatly enhanced by the presence of an Asp in the metal-binding motif due to the ability of the carboxylate group of this residue to coordinate the metal. The Zn(II) site in the MBD seems highly fluxional, which must be important for trafficking this metal and for other potential roles that the ligands, and particularly the Asp residue, may need to perform. The residues in the M/IXCXXC metal-binding motif of copper and zinc trafficking proteins have little influence on Cu(I) affinity.

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