Mechanisms of zinc binding to the solute-binding protein AztC and transfer from the metallochaperone AztD

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Bacteria can acquire the essential metal zinc from extremely zinc-limited environments by using ATP-binding cassette (ABC) transporters. These transporters are critical virulence factors, relying on specific and high-affinity binding of zinc by a periplasmic solute-binding protein (SBP). As such, the mechanisms of zinc binding and release among bacterial SBPs are of considerable interest as antibacterial drug targets. Zinc SBPs are characterized by a flexible loop near the high-affinity zinc-binding site. The function of this structure is not always clear, and its flexibility has thus far prevented structural characterization by X-ray crystallography. Here, we present intact structures for the zinc-specific SBP AztC from the bacterium Paracoccus denitrificans in the zinc-bound and apo-states. A comparison of these structures revealed that zinc loss prompts significant structural rearrangements, mediated by the formation of a sodium-binding site in the apo-structure. We further show that the AztC flexible loop has no impact on zinc-binding affinity, stoichiometry, or protein structure, yet is essential for zinc transfer from the metallochaperone AztD. We also found that 3 His residues in the loop appear to temporarily coordinate zinc and then convey it to the high-affinity binding site. Thus, mutation of any of these residues to Ala abrogated zinc transfer from AztD. Our structural and mechanistic findings conclusively identify a role for the AztC flexible loop in zinc acquisition from the metallochaperone AztD, yielding critical insights into metal binding by AztC from both solution and AztD. These proteins are highly conserved in human pathogens, making this work potentially useful for the development of novel antibiotics.

Zinc is an essential element that is toxic in excess. Therefore, zinc homeostasis is a critical cellular function maintained in bacteria by controlling its import (1) and export (2). High-affinity zinc import is mediated by the ATP-binding cassette (ABC) transporters. These are minimally composed of an intracellular ATPase, transmembrane permease, and extracellular solute-binding protein (SBP). SBPs are extracellular lipoproteins or periplasmic proteins in Gram-positive and Gram-negative organisms, respectively. In either case, the SBP is responsible for binding the appropriate metal with high affinity and specificity and delivering it to the membrane permease for import. Thus, SBPs are required for high-affinity zinc import and survival under extremely zinc-limited conditions such as those imposed on pathogens in animal hosts (3, 4). Importantly, the deletion of zinc SBPs dramatically attenuates virulence among numerous pathogens (5), making them promising targets for the development of novel antibiotics (6). Thus, structural studies aimed at understanding the mechanisms of metal binding and release among this family are of significant interest.

Bacterial ABC transporter systems have most recently been clustered according to structural differences among SBPs, which mirror different substrate types (7). SBPs for zinc belong to cluster A-I, which also includes proteins that transport iron and manganese. Phylogenetic analysis further divides cluster A-I proteins into three main groups (8). These groups differ in terms of metal specificity and coordination environment, organism type, and the presence and composition of a flexible loop near the metal-binding site (Table 1 and Fig. 1). We recently identified and structurally characterized a zinc-specific SBP called AztC (9) that shares the characteristics of all three groups. AztC clusters most closely with the group III iron/manganese transporters and shares a His3/Asp1 coordination environment observed only for TroA, a group III protein. However, it contains a relatively short disordered loop with several His residues (Fig. 1) and exhibits a roughly 100-fold preference for binding zinc over manganese. AztC homologues are found exclusively in Gram-negative organisms including human pathogens, where metal ligands and loop residues are highly conserved.

Like most ABC transporter genes, AztC is encoded in an operon with the canonical ATPase (aztA) and permease (aztB) genes. However, the azt operon is unusual in that it includes a fourth gene, aztD. AztD is a metallochaperone that transfers zinc directly to AztC through a specific, associative mechanism (10). A stable complex is not formed, but zinc transfer is directional and essentially stoichiometric. AztD is highly conserved in azt operons, further suggesting that these may represent a distinct subfamily of bacterial ABC zinc transporter systems.

Metallochaperones to SBPs are rare but not unprecedented. NMR studies show that zinc is transferred directly to AdcAll...
(group Ia) from polyhistidine triad (Pht) proteins (11), chaperones that are essential for transport through AdcAll (12). The mechanism of transfer is unknown. ZnT is a zinc-binding protein induced during zinc limitation and required for optimal growth under these conditions (13–16). It forms a complex with group II SBP ZnuA that is dependent on the presence of zinc (15, 17). Zinc transfer is proposed to occur via the His-rich flexible loop of ZnuA, which projects into the metal-binding cleft of ZnT (17). However, although formation of a stable complex is dependent on the presence of the loop, metal transfer from ZnT to ZnuA has never been demonstrated. Finally, AztD shares no sequence homology with either the Pht proteins or ZnT, making its mechanism of metal transfer an open question.

AztC can acquire zinc either from solution or from the metallochaperone AztD. In this work, we sought to determine the mechanisms of both processes. Crystal structures for holo- and apoAztC reveal significant structural changes accompanying zinc binding. Further, the new holo-structure at improved resolution allowed for the complete modeling of the flexible loop in AztC. To our knowledge, this makes it the first zinc-specific SBP for which the flexible loop feature has been structurally defined. The function of the flexible loop and associated residues in zinc binding from solution and from AztD were also evaluated. The results allow us to propose a mechanism for zinc binding in AztC that is distinct from those of the other cluster A-I SBPs. In addition, this work conclusively determines a function for the AztC flexible loop in zinc acquisition from a metallochaperone AztD.

Results

Comparison of holo- and apoAztC structures

Both apo- and holoAztD crystallized under the same conditions, although in different space groups (Table 2). Both exhibit non-crystallographic symmetry, with two copies in the asymmetric unit (Fig. 2). Residues 25–309 comprise the mature protein after cleavage of the N-terminal periplasmic localization domain for the AztC flexible loop in zinc acquisition from a metallochaperone AztD.

Table 1

| Group | Metal | Coordination | Gram | Flexible loop |
|-------|-------|--------------|------|---------------|
| Ia    | Zn$^{2+}$ | 3 His, 1 Glu | + | Short, no His |
| Ib    | Zn$^{2+}$ | 3 His, 1 Glu | + | Short, His |
| II    | Zn$^{2+}$ | 3 His, 1 Glu/H$_2$O | – | Long, His-rich |
| III   | Mn$^{2+}$/Fe$^{2+}$/Fe$^{3+}$ | 2 His, 1 Asp, 1 Glu | +/- | None |

Table 2

| Data collection, processing, and refinement statistics |
|------------------------------------------------------|
| **Holo-WT AztC** | **Apo-WT AztC** | **Holo-ΔD-loop AztC** |
|--------------------------------|----------------|------------------|
| **Data collection** | | |
| Space group | C2 | P$_2_1$ | P$_2_1$ |
| Unit cell parameters | | | |
| a, b, c (Å) | 104.8, 72.4, 105.6 | 105.6, 108.3, 110.5 | 105.6, 108.3, 110.5 |
| α, β, γ (°) | 90.0, 96.6, 90.0 | 91.0, 111.0, 90.0 | 90.0, 110.7, 90.0 |
| Resolution range (Å) | 48.9–2.30 | 45.8–2.03 | 45.8–2.30 |
| No. of reflections (measured/unique) | 126,304/34,922 | 177,847/47,607 | 160,608/38,783 |
| R$_{merge}$ | 0.05 (0.45) | 0.03 (0.56) | 0.07 (0.46) |
| R$_{free}$ | 12.2 (2.2) | 20.3 (2.4) | 18.6 (2.3) |
| Completeness (%) | 99.5 (99.8) | 99.6 (99.7) | 100.0 (99.9) |
| Redundancy | 3.6 (3.7) | 3.7 (3.6) | 4.1 (3.9) |
| **Refinement statistics** | | | |
| Resolution (Å) | 48.9–2.30 | 45.8–2.03 | 46.4–2.20 |
| R$_{free}$/R$_{free}$ | 21.7/24.1 | 16.7/20.1 | 15.9/20.0 |
| **Number of atoms** | | | |
| Protein | 4,188 | 4,012 | 4,017 |
| Zinc | 2 | 0 | 2 |
| Water | 51 | 136 | 171 |
| Other | 0 | 26 | 12 |
| r.m.s. deviations | | | |
| Bond lengths (Å) | 0.006 | 0.019 | 0.019 |
| Bond angles (°) | 0.89 | 1.90 | 1.95 |
| Ramachandran statistics | | | |
| Allowed (%) | 98 | 98 | 98 |
| Outliers (%) | 2 | 2 | 2 |
| Average B-factor (Å$^2$) | 53.1 | 57.8 | 51.0 |

Figure 1. A portion of the complete multiple sequence alignment of cluster A-I SBPs highlighting the flexible loop region. Organism abbreviations are *Ecoli*, *Escherichia coli*; *Sent*, *Salmonella enterica*; *Pden*, *Paracoccus denitrificans*; *Syn*, *Synechocystis* 6803; and *Spne*, *Streptococcus pneumoniae*. Flexible loop regions as determined by the absence of electron density in crystal structures 2OSV (41) (E. coli ZnuA), 2XY4 (42) (S. enterica ZnuA), 1PQ4 (43) (Synechocystis ZnuA), and 3CX3 (8) (Synechocystis MntC) are shown in red letters. Predicted flexible loop regions are shown in green letters. Residues deleted in the ΔD-loop mutant of *P. denitrificans* AztC are underlined. Subgroup designations as described in the Introduction and in Table 1 are indicated on the right. The His and Trp residues, indicated by asterisks, are found in the metal-binding site and are absolutely conserved among cluster A-I SBPs.
Mechanisms of zinc binding to AztC

depending on which chains are aligned. The most significant structural differences between apo and holo occur in α-helices and loops near the zinc-binding site (Fig. 3). Specifically, helices 3, 7, and 8 and the Z-loop are displaced away from the zinc-binding site in the apo-structure. Features more removed from the zinc site, including the long connecting helix and nearby helices and β-sheets, are nearly superimposable in apo- and holo-structures.

The presence of zinc in holoAztC is confirmed by strong anomalous difference density (Fig. 4A). Zinc is directly coordinated by His61, His138, His204, and Asp279 with bond lengths ranging from 2.0 to 2.3 Å (Table 3). The Z-loop sits directly over the zinc ion, contributing an additional electrostatic interaction at 3.6 Å from the carbonyl oxygen of Val224 (Table 3 and Fig. 4B). This interaction likely stabilizes the position of the Z-loop, as no density was observed for it in one chain of the apo-struc-
ture and in the other it occupies a completely different orientation than in the holo-structure (Figs. 2–4). As expected for the apo-protein, no significant anomalous difference density is observed at the zinc site in apoAztC (Fig. 4C). Further, the zinc ligands are significantly repositioned, particularly His204 and Asp279, in which the side chains rotate away from the zinc site.

These 2 residues (His204 and Asp279) engage new interactions in the apo-state (Fig. 4D). The His204 side chain makes a hydrogen bond at 3.1 Å with the carbonyl oxygen of Ser225 in the B-chain, resulting in significant displacement of the Z-loop.

Although insufficient density was observed in the A-chain to model the Z-loop, His204 is identically positioned in both chains. Asp279 rotates away from the zinc site and, along with Ser58, Thr60, and Asn254, creates a binding site for an atom (Fig. 4, D–F). It is possible that this atom represents a solvent molecule in multiple orientations. However, it was modeled as sodium, because the coordination number, bond lengths, preference for oxygen ligands, and lack of anomalous difference density are all consistent with sodium (18) (Fig. 4, E and F, and Table 3). The putative sodium site is somewhat labile as demonstrated by displacement of the Ser58 side chain in the B-chain (Fig. 4E) by a water molecule in the A-chain (Fig. 4F). Nevertheless, the positions of the other ligands and the general arrangement of the site is conserved in both chains (Table 3).

The reorientation of His204 and the formation of the sodium site appear to drive the larger conformational changes upon zinc loss (Fig. 5). When His204 rotates out of the zinc site to hydrogen bond with Ser225, the Z-loop and its adjacent helix 7 are pushed outward. Asn254 moves to bind sodium in the apo-structure, reorienting this loop and helix 8. Hydrophobic side chains are packed between these two helices, causing them to move as a single unit. Finally, the movement of Ser58 and Thr60 to form the sodium-binding site significantly displaces this loop, which is N-terminal to helix 2 and mediates displacement of it and helix 3. The net effect of these conformational changes is a more open arrangement around the zinc site in the apo-structure relative to the holo-structure (Fig. 5).

The D-loop structure of holoAztC

The D-loop from each chain of the holo-structure and one chain of the apo-structure exhibits continuous electron density for backbone atoms. However, we chose to focus on that from the holo-B-chain, as it allows unambiguous modeling of side chains as well (Fig. 6A). This is likely due to hydrogen bond interactions between the phenol groups of Tyr121 and Tyr123, and several surface residues on the A-chain (Fig. 6B). Aromatic stacking interactions between these Tyr residues and Phe-130

**Table 3**

| Metal–ligand bond distances in apo- and holo-WT AztC |
|-----------------------------------------------------|
| Distances in the A-chain are listed first followed by the B-chain. N.O., indicates that this interaction was not observed. |
| Interaction | HoloAztC | Interaction | ApoAztC |
| His^{61} Ne-Zn | 2.1, 2.0 | Ser^{58} O-Na | N.O., 2.6 |
| His^{138} Ne-Zn | 2.2, 2.3 | Ser^{58} O-Na | 3.6, 3.2 |
| His^{204} Ne-Zn | 2.3, 2.3 | Thr^{60} O-Na | 2.5, 2.3 |
| Asp^{279} O-Zn | 2.0, 2.0 | Asp^{254} O-Na | 2.6, 2.7 |
| Val^{224} O-Zn | 3.6, 3.6 | Asp^{254} O-Na | 2.2, 2.3 |
| Asp^{279} O-Zn | 2.7, 2.7 | H_{2}O-Na | 2.6, 2.5 |

**Figure 4. The zinc- and sodium-binding sites of holo- and apoAztC.** A and B, holoAztC highlighting zinc and its ligands. C and D, apoAztC highlighting former zinc ligands and sodium ligands. E and F, closer view of the sodium-binding site for the B-chain (E) and A-chain (F) of apoAztC. Electron density for a composite omit map at 1.0 σ is shown as blue mesh, and anomalous difference density at 5.0 σ is shown as orange mesh. Hydrogen bond or metal–ligand interactions are shown as dotted black lines. Metal ions are shown as spheres colored according to the element.

**Figure 5. Structural differences between apoAztC (blue) and holoAztC (green).** Metal ligand residues are shown as sticks, and metals are shown as spheres colored according to the element. Important secondary structures are also indicated.
may also contribute to stabilizing this conformation. Flexibility is conferred by the Gly residues that flank the loop (Gly^{117}–Gly^{119} and Gly^{133}). A Gly residue is also found at the very end of the loop (Gly^{126}) allowing for the tight turn at this position. The side chains of all three His residues of the D-loop (His^{120}, His^{122}, and His^{131}) are on the same face of the loop and spatially close, with His^{131} occupying two alternate conformations (Fig. 6). This arrangement could easily represent a metal-binding site, although no anomalous difference density was detected at this position and binding assays indicate that AztC only binds a single zinc ion with high affinity. Nevertheless, it suggests that these His residues could temporarily coordinate zinc and convey it to the high-affinity site. This would require an open zinc-binding site with a displaced Z-loop, as indicated for the apo-structure.

The structure of a deletion mutant lacking His^{120}–Ala^{132} (called ΔD-loop AztC throughout) was also determined, to evaluate whether the D-loop has any impact on the overall structure (Fig. 7 and Table 2). Although the mutant crystallized in a different space group from WT holoAztC, the structure is essentially identical with r.m.s. deviations = 0.28 - 0.33 Å². Zinc is bound at full occupancy in the same coordination environment as WT. The deletion of the D-loop leaves a run of 4 Gly residues near the zinc site, which exhibit clear, continuous electron density (Fig. 7 A). This suggests that the D-loop is a highly flexible structure with no significant influence on the overall structure or the coordination environment of zinc in AztC.

Function of the AztC loop structures

The structural data presented above suggest that the D-loop could temporarily coordinate zinc and deliver it to the open binding site of the apo-structure. Upon binding, the zinc ligands would swing back into the site, closing helices 3, 7, and...
and the Z-loop down over the metal. To test the functions of these two loops, a ΔZ-loop mutant lacking residues 222–229 was also generated, and its zinc-binding properties were compared with both the WT and ΔD-loop AztC. Like the WT, both mutants were purified with approximately stoichiometric zinc, >95% of which could be removed by dialysis at low pH as determined by inductively coupled plasma-optical emission spectrometer (ICP-OES). Competition assays with the fluorescent zinc probe Mag-Fura 2 (MF2) (Fig. 8) demonstrate that the D-loop mutant binds a single zinc ion with $K_d = 0.2 \pm 0.1$ nM (Fig. 8B). This is essentially identical to the WT, which binds a single zinc ion with $K_d = 0.3 \pm 0.1$ nM (9). The Z-loop mutant actually appears to exhibit a somewhat higher affinity than the WT (Fig. 8C). However, extremely high affinities are difficult to measure accurately given the mid-nanomolar affinity of MF2 (19). Therefore, we assigned a value of <0.2 nM to the ΔZ-loop AztC $K_d$. In any case, it is clear that neither of the mutations inhibits zinc binding from solution.

WT AztC exhibits a strong increase in intrinsic protein fluorescence upon zinc binding, reaching roughly a 2-fold emission intensity upon saturation with 1 zinc equivalent (10). This property is maintained in each of the deletion mutants (Fig. 9). Each deletion mutant is saturated at ~1 equivalent of added zinc, although the net increase in fluorescence is somewhat less for ΔZ-loop AztC. Nevertheless, these data are consistent with MF2 assays indicating that deletion of either loop does not impact the ability of the AztC to bind zinc from solution.

The change in intrinsic fluorescence allows for the qualitative determination of zinc transfer from the chaperone AztD (9) (Fig. 9). Zinc transfer is determined by the increase in AztC fluorescence intensity at 315 nm, as holoAztD is added to a slight excess in terms of zinc. In this case, holoAztD refers to this protein as isolated, which contained a 0.73–0.89 molar equivalent of zinc. WT AztC shows large increases in fluorescence due to the combined effects of zinc binding and the intrinsic fluorescence contributed from added AztD protein (9). Importantly, the addition of excess ZnCl$_2$ caused no further changes, indicating that the AztC is saturated with zinc transferred from AztD. A similar result is observed for ΔZ-loop AztC. In contrast, ΔD-loop AztC exhibits very little change over the course of the titration and a dramatic change upon the addition of excess zinc. Thus, although neither loop is required for the binding of zinc from solution, the D-loop is required for the efficient acquisition of zinc from AztD.

The 1 Asp and 3 His residues of the D-loop (Fig. 1) are conserved among AztC homologues and could serve as zinc ligands during transfer. Additionally, these 3 His residues are oriented in such a way as to generate a potential metal-binding site in one chain of the holo-WT AztC structure. Tyr$^{121}$, which is between 2 His residues, is also conserved and may participate in specific interactions between AztC and AztD. To evaluate the role of the conserved loop residues, each was mutated to alanine and intrinsic fluorescence assays were repeated. All mutants exhibited excellent stability and solubility. Zinc binding from solution was confirmed for all mutants by intrinsic fluorescence, although the magnitude of the fluorescence changes was somewhat variable (Fig. 9). However, significant differences were observed in terms of the ability of the mutants to acquire zinc from AztD (Fig. 9). The results indicate that mutation of any of the 3 His residues (His$^{120}$, His$^{122}$, and His$^{131}$) results in a protein that cannot be saturated with zinc transferred from AztD.
On the other hand, Y121A and D125A acquired zinc to saturation from the chaperone. From this we concluded that each of the 3 His residues of the loop is required for efficient zinc acquisition from AztD, whereas Tyr121 and Asp125 are dispensable in this process.

Figure 9. Intrinsic fluorescence of zinc binding and transfer from AztD. Fluorescence emission ($\lambda_{ex} = 278$ nm) of $10 \mu M$ apoAztC mutants titrated with $2 \mu M$ additions of ZnCl$_2$ (left two columns) or holoAztD (right two columns). Fluorescence intensity increases up to saturation at $1$ equivalent of added zinc. Titrations with holoAztD (solid lines, increasing in intensity) were followed by the addition of $20 \mu M$ ZnCl$_2$ to determine saturation (dashed line). Each plot is representative of an experiment performed at least twice.

Discussion

The mechanisms of metal binding are inferred by comparing the apo- and holo-structures for several cluster A-I SBPs (20–25). The type and degree of structural changes accompanying metal binding vary significantly. All exhibit some reorientation of His$^{61}$ (AztC numbering) away from the binding site. Modest reorientations of loops and helices near the metal-binding site, similar to what we see in AztC, have also been observed in ZnuA from *Escherichia coli* (cluster II) and PsaA from *Streptococcus pneumoniae* (cluster III). To date, the formation of a sodium-binding site in the apo-structure of a cluster A-I SBP that reorients a zinc ligand is unique to AztC. This suggests that the presence of a monovalent cation may be important for metal release. It will be of interest to evaluate the impact and specificity of sodium binding on zinc coordination and release in AztC.

AztC shares several significant structural similarities with TroA, despite relatively weak sequence identity (16%). Although it belongs to cluster III, TroA exhibits only marginal specificity for manganese over zinc *in vitro* (26–28). Its physiological substrate is also somewhat controversial, although one study suggests a predominant role in manganese transport in *Streptococcus suis* (29). In any case, TroA from both *S. suis* (28) and *Treponema pallidum* (30) exhibit coordination geometries identical to that of AztC. The Z-loop structure and sequence are also highly conserved, including an interaction between the carbonyl oxygen atom of Val$^{219}$ (*Tp* TroA) or Val$^{217}$ (*Ss* TroA) and bound zinc at 3.7–3.9 Å. No other cluster A-I SBP solved to date shares this feature. However, the structures of apo (22) and holo (30) *Tp* TroA are nearly identical, with none of the extensive remodeling near the metal-binding site seen in AztC.
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Intriguingly, Asn254 and Ser58, in which the side chains serve as sodium ligands in the apoAztC structure, are not conserved in TroA. Thus, the formation of the sodium site in AztC may drive the larger conformational changes that are absent in TroA. How or even if these contribute to the improved specificity of AztC for zinc over manganese is uncertain, but there are few other structural differences between AztC and TroA to account for this difference.

The combination of structural and fluorescence data allowed us to propose mechanisms for zinc binding to AztC from solution (Fig. 10) and from AztD. The former does not require the flexible loop. Rather, zinc can access the open binding site directly, coordinating with His120, His122, and His131. The data suggest that the D-loop can access the zinc site of AztD, coordinate the metal via the His residues, and deliver it to the high-affinity site of AztC. The flexibility prerequisite for this activity is conferred by Gly residues at the loop hinge regions and at the end of the loop. This mechanism is consistent with the comparable zinc-binding affinities of AztC (9) and AztD (10), which indicate that the transfer process cannot be thermodynamically driven. It is also consistent with the observation that zinc transfer is still highly efficient in the presence of 1 mM EDTA, indicating that zinc is never lost to solution (10).

The Z-loop appears to have no impact on zinc binding either from solution or from AztD. If anything, its presence somewhat decreases zinc affinity. It is highly flexible in the apo-structure and may simply need to be out of the way for efficient zinc binding. However, it may have a role in permease recognition and subsequent metal release. Experiments to test this hypothesis will require an evaluation of ΔZ-loop AztC function in vivo, experiments that are under way in our laboratory.

Our work here highlights some of the unusual structural and functional properties of the AztABCD transporter system. AztC is somewhat of a hybrid of cluster A-I SBPs, bearing characteristics of group Ib (zinc specificity and a short, His-containing loop), group II (Gram-negative only), and group III (TroA-like coordination) as well as some that appear unique (sodium-binding site in apo and zinc transfer from AztD). Thus, AztC expands the repertoire of metal-binding mechanisms of the cluster A-I SBPs. Given the conservation of the azt operon in human pathogens, it is hoped that continued study of this system may also yield therapeutically valuable insights.

**Experimental procedures**

**Expression and purification of proteins**

WT AztC (9) and AztD (10) were expressed and purified as described previously. The AztC loop deletions lacking residues 120–132 and 222–229 as well as point mutants H120A, Y121A, H122A, D125A, and H131A were generated using the Q® site-directed mutagenesis kit (New England Biolabs). All mutants were confirmed by plasmid sequencing. The mutant proteins were expressed and purified as described for the WT, except that we lysed cells in 50 mM Tris, pH 8.0, 150 mM NaCl, and 0.1 mM DTT rather than performing periplasmic extraction to maximize yields. The presence and purity of all proteins were confirmed using SDS-PAGE, and concentration was measured using an extinction coefficient of 19,691 M⁻¹ cm⁻¹ as determined previously for WT AztC.

**Zinc quantitation and generation of apo-proteins**

Apo-proteins were generated by dialysis at 4 °C against two changes of 500 ml of 50 mM NaOAc buffer, pH 4.5, 50 mM EDTA, and 150 mM NaCl. This was followed by dialysis against two changes of 500 ml of 50 mM Tris buffer, pH 8.0, 150 mM NaCl, and 3.4 g/liter Chelex. Protein samples at a concentration of 10–20 μM were digested in 4 M HNO₃ overnight at 70 °C and diluted 2.5-fold with MilliQ water prior to metal analysis. For buffer blanks, 2.5 ml was combined with 0.5 ml of concentrated HNO₃ and digested overnight at 70 °C. The metal content was quantified using a PerkinElmer 2100 DV ICP-OES calibrated with a multielement standard (Alfa Aesar) at a wavelength of 213.857 nm. All samples were run in triplicate.
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Mag-Fura 2 competition assay

Zinc-binding affinities were measured using a MF2 competition assay derived from Golynskiy et al. (19) as described previously (9, 10). All fluorescence measurements were made using a Varian Cary Eclipse fluorescence spectrophotometer with entrance and exit slits set to 10 nm. The protein concentration was measured before each experiment, and MF2 concentration was determined using an extinction coefficient at 369 nm of 22,000 M$^{-1}$cm$^{-1}$ (19). In each experiment, 1.0 μM apo-protein and 0.5 μM MF2 were titrated with increasing concentrations of ZnCl$_2$, keeping the total volume of titrant added to less than 10% v/v. Fluorescence excitation spectra were scanned from 250 to 450 nm with monitoring emission at 505 nm. Experiments were performed in triplicate, and the fluorescence intensities at A$_{ex}$ = 330 nm were fit using the program DYNAFIT (31, 32) with scripts adapted from Golynskiy et al. (19). Prior to each series of experiments, the affinity of MF2 for zinc in our buffer system was determined using DYNAFIT and used in our calculation of protein-binding affinity. The MF2 affinity value ($K_a$) varied from 92 to 141 nM.

Crystallization and structure determination

WT apoAztC and ΔD-loop AztC were crystallized under the same conditions used for holo-WT (9). Crystals were cryoprotected with a 1:1 mixture of paratone/paraffin prior to cryocooling in liquid nitrogen. Diffraction data, collected at 100 K on beamline 8.2.2 or 5.0.2 at the Advanced Light Source at Berkeley National Laboratory, were indexed, integrated, and scaled with HKL2000 (33) or XDS (34, 35). The WT AztC structure (9) was used as the search model for molecular replacement using Phaser-MR (36). Manual model building was done in Coot (37), and further rounds of refinement were done using REFMAC (38) or Phenix (39). The latter was also used to generate anomalous difference maps and composite omit maps (40). The coordinates of WT holoAztC, WT apoAztC, and ΔD-loop AztC have been deposited in the Protein Data Bank with entry codes 5W57, 5W56, and 5KZJ, respectively. Figs. 2–7 and 10 were prepared using PyMOL (44), which was also used for pairwise structural alignments.

Zinc binding and transfer by intrinsic fluorescence

All fluorescence measurements were made using a Varian Cary Eclipse fluorescence spectrophotometer as described previously (10). AztC proteins at 10 μM were titrated with 2 μM ZnCl$_2$ or holoAztD per addition. The zinc content of all AztD preparations was measured by ICP-OES allowing for determination of fluorescence intensity changes versus added zinc. In titrations of apoAztC with holoAztD, a 5–15 min of equilibration was allowed between measurements. After addition of holo-AztD to 16 μM, 20 μM of ZnCl$_2$ was added to determine AztC saturation.

Author contributions—D. P. N. and H. R. performed and analyzed the fluorescence experiments. D. A. and E. T. Y. crystallized the proteins, collected diffraction data, and built and refined the crystallographic models. S. F. and D. P. N. generated and purified the WT and mutant proteins. E. T. Y. conceived and coordinated the study and wrote the paper.

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