The Mechanism of Cu\(^{+}\) Transport ATPases

INTERACTION WITH CU\(^{+}\) CHAPERONES AND THE ROLE OF TRANSIENT METAL-BINDING SITES*

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Background: Cytoplasmic chaperones deliver Cu\(^{+}\) to P\(_{1B}\)-ATPases for outward transport.

Results: Alterations of an invariant electropositive platform, metal ligating residues on the ATPase, and electronegative surface of the chaperone abolish Cu\(^{+}\) transference. Alterations of an invariant electropositive platform, metal ligating residues on the ATPase, and electronegative surface of the chaperone abolish Cu\(^{+}\) transference. This docking likely places the chaperone into a given compartment, the metal accesses (and probably leaves) transmembrane transporters via chaperone-mediated processes (9–12).

Conclusion: Electrostatics drives Cu\(^{+}\) chaperone/ATPase platform interaction. Ligand exchange by carboxyl and thiol groups enables Cu\(^{+}\) release from the chaperone.

Significance: The mechanism of transition metal access to transmembrane transport sites is described.

Cu\(^{+}\)-ATPases are membrane proteins that couple the hydrolysis of ATP to the efflux of cytoplasmic Cu\(^{+}\). In cells, soluble chaperone proteins bind and distribute cytoplasmic Cu\(^{+}\) deliv- ering the ion to the transmembrane metal-binding sites in the ATPase. The structure of *Legionella pneumophila* Cu\(^{+}\)-ATPase (Gourdon, P., Liu, X. Y., Skjorringe, T., Morth, J. P., Moller, L. B., Pedersen, B. P., and Nissen, P. (2011) *Nature* 475, 59–64) shows that a kinked transmembrane segment forms a “platform” exposed to the cytoplasm. In addition, neighboring invariant Met, Asp, and Glu are located at the “entrance” of the ion path. Mutations of amino acids in these regions of *Archaeoglobus fulgidus* Cu\(^{+}\)-ATPase CopA do not affect ATPase activity in the presence of Cu\(^{+}\) free in solution. However, Cu\(^{+}\) bound to the corresponding chaperone (CopZ) could not activate the mutated ATPases, and in parallel experiments, CopZ was unable to transfer Cu\(^{+}\) to CopA. Furthermore, mutation of a specific electronegative patch on the CopZ surface abolishes the ATPase activation and Cu\(^{+}\) transference, indicating that the region is required for the CopZ-CopA interaction. Moreover, the data suggest that the interaction is driven by the complementation of the electropositive platform in the ATPase and the electronegative Cu\(^{+}\) chaperone. This docking likely places the Cu\(^{+}\) proximal to the conserved carboxyl and thiol groups in the entrance site that induce metal release from the chaperone via ligand exchange. The initial interaction of Cu\(^{+}\) with the pump is transient because Cu\(^{+}\) is transferred from the entrance site to transmembrane metal-binding sites involved in transmembrane translocation.

Copper is an essential micronutrient required for many biological processes. It plays important catalytic roles as a prosthetic group in enzymes such as copper/zinc superoxide dismutase, cytochrome *c* oxidase, and tyrosinase (1). Copper is also toxic to cells because it participates in Fenton reactions and interferes in [Fe-S] protein assembly (2, 3). Therefore, Cu\(^{+}\) homeostasis is tightly controlled by transmembrane transporters, metallochaperone proteins, transcriptional regulators, and abundant small molecules that bind Cu\(^{+}\) with high affinity (4–8). This results in low intracellular Cu\(^{+}\) quotas (10–100 \(\mu\)M) and the formal absence of free cytoplasmic Cu\(^{+}\) (4). As a corollary, to prevent release of free Cu\(^{+}\) into a given compartment, the metal accesses (and probably leaves) transmembrane transporters via chaperone-mediated processes (9–12).

P\(_{1B}\)-type Cu\(^{+}\)-ATPases drive the efflux of cytoplasmic Cu\(^{+}\) (13–15). These integral membrane proteins use the energy provided by ATP hydrolysis to transport the metal across membranes. Mutation of coding genes in eukaryotes leads to serious imbalances in copper homeostasis (5, 8). For instance, ATP7A and ATP7B are human Cu\(^{+}\)-ATPases associated with Menkes and Wilson diseases, respectively (5). In bacteria, Cu\(^{+}\)-ATPases are responsible for maintaining cytoplasmic copper levels, and their participation in periplasmic metalloprotein assembly has been postulated (6, 14, 15). The metal translocation mechanism essentially follows an E1/E2 Albers-Post cycle with the hallmark metal-dependent catalytic phosphorylation of an invariant Asp (DKTGT) (13, 15, 16). Within this framework, characterization of model systems has shown the ATPase activation by alternative substrates (Cu\(^{+}\)/Ag\(^{+}\)), regulation by cytoplasmic N-terminal metal binding domains (N-MBD), a stoichiometry of two Cu\(^{+}\) ions transported by ATP hydrolyzed, and the outward Cu\(^{+}\) transport (13–16).

The structure of Cu\(^{+}\)-ATPases consists of eight transmembrane helices (TM) and several cytoplasmic domains: the actuator domain between TM4 and TM5, the ATP-binding and phosphorylation domains between TM6 and TM7, and cytosolic N-MBDs. Bacteria and archaea contain one or two \(~7-kDa\) N-MBDs, but eukaryotic proteins can have up to six of these well characterized regulatory domains (13, 17). The ATPase N-MBDs sense Cu\(^{+}\) by exchanging metal with Atx1-like Cu\(^{+}\)-metallochaperones, such as CopZ in bacterial systems (4, 10–100 \(\mu\)M).

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2 The abbreviations used are: N- and C-MBD, N- and C-terminal metal-binding domain; DDM, n-dodecyl-β-D-maltopyranoside; TM, transmembrane segment; TM-MBS, transmembrane metal-binding site; Strep, streptavidin.
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18–20). Truncation or mutation of N-MBDs results in functional CopAs, although these mutants show altered kinetics (21–23). N-MBDs appear to regulate transport rates by interacting with the ATP-BD in a Cu\(^{+}\)-dependent manner (24–26). The electrostatic nature of the interaction between electropositive residues on the surface of the yeast chaperone Atx1 (27, 28) and the electronegative residues on the cytosolic regulatory N-MBD segment of the Ccc2 ATPase has been analyzed (29). Mutation of Lys residues on the surface of Atx1 reduces the Cu\(^{+}\)-dependent complex formation between Atx1 and the ATPase (27), thus pointing out the requirement of complementary charged side chains. Upon this initial electrostatic docking, other interactions are established to direct the chaperone Cu\(^{+}\)-binding residues toward N-MBD and the subsequent ligand exchange (30).

The defining characteristic of Cu\(^{+}\)-ATPases is their transmembrane metal-binding sites (TM-MBSs) responsible for metal translocation (17, 31, 32). Conserved amino acids present in TM6 (two Cys), TM7 (Asn and Tyr), and TM8 (Met and Ser) constitute two trigonal Cu\(^{+}\) TM-MBSs. Occupancy of both sites is required for ATP hydrolysis and consequent ion translocation. These sites bind Cu\(^{+}\) with high (iM) affinities and, as in the case of other P-type ATPases, in the absence of ATP, the metal substrate-enzyme complex (metal occluded conformation) can be isolated and characterized. However, distinct from well characterized alkali and alkali-earth substrates of P\(_2\)-ATPases, in vivo Cu\(^{+}\) does not access the enzyme in a free (hydrated) form but bound to soluble chaperones (4, 10, 20). We have shown that the Cu\(^{+}\)-chaperone delivers Cu\(^{+}\) directly to the TM-MBS (i.e., independent of the N-MBDs) and proposed that the metal transfer requires the initial docking of the chaperone with the ATPase in close proximity to the TM-MBS (10, 24). This interaction would allow Cu\(^{+}\) ligating groups in the ATPase to substitute those in the chaperone, enabling Cu\(^{+}\) transfer via ligand exchange. Implicit in this model was the specific recognition of the interacting proteins contributing to the enzyme selectivity mechanism and to constant metal sequestration (11, 15).

The crystal structure of Legionella pneumophila CopA (LpCopA), a typical Cu\(^{+}\)-ATPase, was obtained by locking the enzyme in a Cu\(^{+}\)-free E2-AlF\(_4\) conformation (33). This described the arrangement of the TM domains and confirmed the proximity of amino acids forming the TM-MBSs. Interestingly, the structure revealed a novel structure formed by a kink in the second TM that generates a “platform” at the border of the membrane inner leaflet (see Fig. 1). Residues in this region are not fully conserved, but a preponderance of electropositive residues in the region is evident Cu\(^{+}\)-ATPases sequences (see Fig. 1C). In addition, Gourdon et al. (33) noticed a putative Cu\(^{+}\) “entrance” site at the intracellular interface, constituted by three fully conserved residues (Met, Asp, and Glu). Taking into account the electropositive exposed surface of the platform region and the electronegative area present in Cu\(^{+}\) chaperones (see Figs. 1A and 4) (19), a plausible model was postulated where the electrostatic-driven docking allows the metal exchange facilitated by polar residues in the ATPase.

The Archaeoglobus fulgidus CopA-CopZ provides a framework to test these ideas, because this Cu\(^{+}\)-ATPase (AfCopA) and its corresponding Cu\(^{+}\) chaperone (AfCopZ) have served as models for previous mechanistic studies (11, 15, 16, 18, 34). The description of their interaction provided a basis for our current understanding of Cu\(^{+}\) access to transmembrane transporters (10, 24). Molecular modeling has showed the Cu\(^{+}\)-dependent favorable polar binding energy of the docking of AfCopZ-Cu\(^{+}\) to the platform region in AfCopA (11) (see Fig. 1, A and B). Based on the LpCopA structure, we hypothesize that in A. fulgidus the Cu\(^{+}\) delivery from AfCopZ to AfCopA begins with the electrostatic interaction between the negatively charged residues in AfCopZ (Asp-145, Glu-190, Glu-193, and Asp-205) (see Fig. 4A) and the positively charged residues in the platform of AfCopA (Lys-145, Arg-152, Arg-153, and Arg-154) (see Fig. 1B). Subsequently, the conserved Met-158, Glu-205, and Asp-336 (see Fig. 1B) establish a transient initial binding of Cu\(^{+}\) to remove the ion from the chaperone, which then moves into the TM-MBS. In this work, using site-directed mutagenesis, enzymatic analysis, and Cu\(^{+}\) transfer determinations, we provide experimental evidence supporting this model and show the relevance of these structures for the mechanism of Cu\(^{+}\) transport by P\(_{1\beta-1}\)-ATPases.

EXPERIMENTAL PROCEDURES

Bioinformatic Analyses—Protein homology modeling was performed with RasWin Molecular Graphics software (V. 2.7.5.2). AfCopA and AfCopZ C-terminal chaperone domains were modeled using LpCopA (Protein Data Bank code 3RFU) and Enterococcus hirae CopZ (Protein Data Bank code 1CPZ) as corresponding templates. A pool of 380 Cu\(^{+}\)-ATPase protein sequences of bacterial genomes was used to analyze the conservation of the platform region and Cu\(^{+}\) entrance sites. Sequences were aligned using ClustalW2 (35), and the results were visualized using the Multiple Em for Motif Elicitation software (36).

Cloning, Protein Expression, and Purification—AfCopA cDNA lacking the N-MBD and C-MBD coding regions (ΔN, C-CopA) (10), was ligated into pEXP-NT vector, which adds an N terminus His\(_6\) tag sequence (Invitrogen). ΔN,C-CopA was used as a template to introduce the mutations coding for the single substitutions M158A, M158C, E205A, E205C, M158A, M158C, E205A, E205C, D336A, and D336C, and the multiple replacements S139A/G140A and K145A/S149A/R152A/R153A/R154A (PLAT mutant), employing a QuickChange™ site-directed mutagenesis kit (Stratagene). Primers used are listed in Table 1. AfCopZ was previously cloned in pPRIBA1 (IBA), which adds a C-terminal streptavidin (Strep) tag sequence (10). This construct was used as a template for amplifying Ct-CopZ. Ct-CopZ contains the classic Cu\(^{+}\) chaperone domain but lacks a unique iron-sulfur N-terminal domain present in the full-length AfCopZ (24). Bioinformatics analysis of the Ct-CopZ shows the presence of three electronegative patches in its surface. Each of these was modified by introducing simultaneous multiple substitutions: Ct-CopZ M1 (D145A/E190A/E193A/E205A; see Fig. 4A), Ct-CopZ M2 (E179A/D180A/E181A/E182A/E184A; see Fig. 4B), and Ct-CopZ M3 (E161A/E162A/Glu169A; see Fig. 4C). Wild type and mutated ΔN,C-CopA-pEXP-NT, CopZ-pPRIBA1, and Ct-CopZ-pPRIBA plasmids were transformed into Escherichia coli BL21 (DE3) cells. Protein expression of all
ΔN,C-CopA wild-type and mutant proteins was performed according to an autoinducing media protocol (37). AfCopZ wild-type and mutant protein expression was induced for 3 h by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside. Purification of membrane and soluble proteins were carried out as described (10, 18, 34, 38). Solubilized lipid/detergent micellar forms of ΔN,C-CopA and mutant apo forms were loaded with Cu

| Primer name       | Sequence                                                                 |
|-------------------|--------------------------------------------------------------------------|
| M158A forward     | 5’-GCCGAAGGTTTCTGCAGCTCAGCGGCAAG-3’                                      |
| E205A reverse     | 5’-GCCGAAGGTTTCTGCAGCTCAGCGGCAAG-3’                                      |
| D336A forward     | 5’-GCCGAAGGTTTCTGCAGCTCAGCGGCAAG-3’                                      |
| M158C forward     | 5’-GCCGAAGGTTTCTGCAGCTCAGCGGCAAG-3’                                      |
| E205C forward     | 5’-GCCGAAGGTTTCTGCAGCTCAGCGGCAAG-3’                                      |
| S139A/G140A forward| 5’-GCCGAAGGTTTCTGCAGCTCAGCGGCAAG-3’                                      |
| Ct-CopZ forward   | 5’-GCCGAAGGTTTCTGCAGCTCAGCGGCAAG-3’                                      |
| Ct-CopZ reverse   | 5’-GCCGAAGGTTTCTGCAGCTCAGCGGCAAG-3’                                      |
| Ct-CopZ M1F1      | 5’-GCCGAAGGTTTCTGCAGCTCAGCGGCAAG-3’                                      |
| Ct-CopZ M1R2      | 5’-GCCGAAGGTTTCTGCAGCTCAGCGGCAAG-3’                                      |
| Ct-CopZ M2F1      | 5’-GCCGAAGGTTTCTGCAGCTCAGCGGCAAG-3’                                      |
| Ct-CopZ M2R2      | 5’-GCCGAAGGTTTCTGCAGCTCAGCGGCAAG-3’                                      |
| Ct-CopZ M3F1      | 5’-GCCGAAGGTTTCTGCAGCTCAGCGGCAAG-3’                                      |
| Ct-CopZ M3R2      | 5’-GCCGAAGGTTTCTGCAGCTCAGCGGCAAG-3’                                      |
The absence of ΔN,C-CopA in the wash fractions and AfCopZ in the elution fractions was confirmed by SDS/PAGE. Controls were performed where Cu⁺-loaded AfCopZ-Strep or ΔN,C-CopA were subjected to the same procedures individually, i.e., lacking a partner Cu⁺-exchanging protein.

RESULTS

The Role of the Platform Region of Cu⁺-ATPases in the Interaction with Cu⁺ Chaperones—Sequence alignment and homology modeling based on the structure of LpCopA were performed to map the platform region within the AfCopA structure (Fig. 1). The kink of TM2 begins at Ser-139/Gly-140, equivalent to Gly-129 and Gly-130 in LpCopA, and the platform structure exposes the positively charged residues Lys-145, Arg-152, Arg153, and Arg-154 (Fig. 1B). Toward testing the participation of these residues in enzymatic activity but most importantly in Cu⁺ transfer from AfCopZ to AfCopA, they were substituted Ala within the ΔN,C-CopA background (AfCopA lacking the N- and C-MBS). The ΔN,C-CopA construct, although retaining the enzymatic characteristics of the full-length protein, allows the analysis of metal delivery to the TM-MBSs without the interference of metal transfer to regulatory N- and C-MBDs (10, 31). Two proteins were engineered, one simultaneously altering the two amino acids at the kink (S139A/G140A), and the PLAT construct lacking the electro-positive residues at the platform (K145A/S149A/R152A/R153A/R154A). These proteins were heterologously expressed and affinity-purified (10). Fig. 2 shows the SDS/PAGE and immune staining analyses of the resulting proteins. The functionality of these proteins was first evaluated by measuring their ATPase activity and Cu⁺ activation independent of the presence of AfCopZ. In vivo Cu⁺-ATPases are activated by Cu⁺ bound to chaperones; however, in vitro the free ion can directly access the TM-MBS, activate the enzyme, and be transported (15, 16). Table 2 shows that the introduced amino acid replacements did not significantly affect the enzyme $V_{\text{max}}$, the $K_{\text{m}}$ for Cu⁺ activation, or the binding of Cu⁺ to TM-MBSs. These data indicate that the mutated proteins are able to interact with the
Ser-139 and Gly-140 did not prevent platform interaction. These suggest that although the platform is required for the Cu ATPase catalytic cycle, and undergo the conformational changes associated with the substrate (when this is independent of the chaperone CopZ) and undergo the conformational changes associated with the ATPase catalytic cycle.

The role of the platform region as the place of chaperone-Cu\(^{2+}\)-ATPase interaction was tested by measuring the activation of the S139A/G140A and PLAT mutants by saturating concentrations (10 \(\mu\)M) of AfCopZ-Cu\(^{2+}\) (Fig. 3A) (10). Supporting the proposed model, the PLAT mutant was not activated by the Cu\(^{2+}\)-bound chaperone. However, mutations in the nearby Ser-139 and Gly-140 did not prevent \(\Delta N, C\)-CopA activation. These suggest that although the platform is required for the AfCopZ-AfCopA interaction and associated Cu\(^{2+}\) transfer, nearby structural alterations do not affect these events. We have observed that occupancy of both Cu\(^{2+}\) TM-MBSs is necessary for ATPase activation (32) and that chaperone-mediated Cu\(^{2+}\) loading of both sites requires the presence of nucleotide driving the enzyme to an E1-Cu\(^{2+}\) (nucleotide) form (14, 15, 24). Then it could be argued that the lack of PLAT mutant activation might be associated with altered conformations required for binding of the second Cu\(^{2+}\) (rather than chaperone docking).

Testing the interaction and metal transfer independent of enzyme turnover, we analyzed the unidirectional Cu\(^{2+}\) transfer from CopZ-Cu\(^{2+}\) to the \(\Delta N, C\)-CopA mutants (Fig. 3, B and C). To this end, the Cu\(^{2+}\)-loaded Strep-tagged AfCopZ chaperone was incubated with His-tagged apo-\(\Delta N, C\)-CopA mutants. The proteins were separated using a Ni\(^{2+}\) resin, and the resulting fractions were analyzed for protein and Cu\(^{2+}\) content as previously described (10, 24). Fig. 3B shows that Cu\(^{2+}\) eluted associated with \(\Delta N, C\)-CopA wild type and S139A/G140A mutant, but it did not elute with the \(\Delta N, C\)-CopA PLAT mutant, suggesting a direct role of the platform in the interaction among these proteins. Fig. 3C shows the elution of AfCopZ and \(\Delta N, C\)-CopA proteins. Required controls testing the elution of chaperone and ATPase in the absence of the other partner were performed (data not shown). Controls showing no transfer after mutation of amino acids at the TM-MBS have been previously reported (10, 24).

The Exposed Negatively Charged Surface of the Cu\(^{2+}\) Chaperone Is Required for the Interaction with the ATPase—The participation of the CopA electropositive platform in the interaction with the chaperone led us to consider the presence of an electronegative counterpart in the chaperone. Sequence alignment and homology modeling based on the structure of CopZ E. hirae were performed to determine the electronegative exposed regions on A. fulgidus Ct-CopZ. Three major negatively charged patches were identified (Fig. 4). To test the participation of these regions of CopZ in the interaction, Ala mutations were introduced within the Ct-CopZ background (AfCopZ lacking the iron-sulfur N-domain). The A. fulgidus Ct-CopZ construct has been shown to transfer one Cu\(^{2+}\) to TM-MBSs is necessary for ATPase activation (32) and that chaperone-mediated Cu\(^{2+}\) loading of both sites requires the presence of nucleotide driving the enzyme to an E1-Cu\(^{2+}\) (nucleotide) form (14, 15, 24). Then it could be argued that the lack of PLAT mutant activation might be associated with altered conformations required for binding of the second Cu\(^{2+}\) (rather than chaperone docking).

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MBSs of \( \Delta N, C, \text{CopA} \) and fully activate the ATPase (24). Thus, three proteins were engineered, simultaneously mutating the negative residues at the three patches: Ct-CopZ M1 (E190A/E193A/E205A/D145A; Fig. 4 A), Ct-CopZ M2 (E184A/E179A/D180A/E181A/E182A; Fig. 4 B), and Ct-CopZ M3 (E169A/E161A/E162A; Fig. 4 C). Fig. 3 shows the SDS/PAGE and immune staining analyses of the heterologously expressed and affinity-purified proteins (10). None of the mutations affected the stoichiometry of Cu\(^{2+}\) binding to Ct-CopZ (Table 3). The role of the three negatively charged patches in Ct-CopZ as the site of chaperone/Cu\(^{2+}\)-ATPase interaction was evaluated by measuring Cu\(^{2+}\) transfer determinations. As expected, whereas Ct-CopZ M1 was unable to transfer Cu\(^{2+}\) to CopA, the Ct-CopZ M2 and M3 mutants delivered Cu\(^{2+}\) to the TM-MBDs of CopA (Fig. 5). Thus, the data show the specific requirement of certain electronegative patches on CopZ. That is, the alteration of neighboring electronegative areas of the chaperone do not affect Cu\(^{2+}\) transfer and the subsequent ATPase activation. Interestingly, these data are in agreement with previous bioinformatic analysis showing that the electrostatic docking of these proteins relies in a specific interaction between the E190/E193/E205/D145 region of Ct-CopZ (Fig. 4 A) and the electropositive platform of CopA (11).

Invariant Entrance Residues (Met, Glu, and Asp) Are Required for Chaperone-mediated Cu\(^{2+}\) Access to TM-MBS—Sequence comparison and molecular modeling indicate that Met-158, Glu-205, and Asp-336 are invariant amino acids located at the end of the platform in AfCopA, equivalent to those pointed out by Gourdon et al. (33) (Fig. 1 B). Previous studies suggested that no binding of Cu\(^{2+}\) to a site formed by these residues is likely; i.e., stably bound Cu\(^{2+}\) is only associated with the two TM-MBSs (31). Nevertheless, the full conservation of these residues and their metal-binding side chains suggests an important role in the enzyme function. Testing this, they were replaced by Ala or Cys as a conservative substitution in the \( \Delta N, C, \text{CopA} \) background. Fig. 2 shows the resulting purified proteins. As in the case of the platform helix, replacement

FIGURE 3. AfCopZ-Cu\(^{2+}\) interaction with \( \Delta N, C, \text{CopA} \) S139A/G140A and PLAT mutants. A, AfCopZ-Cu\(^{2+}\)-dependent ATPase activity. Wild type and mutated \( \Delta N, C, \text{CopA} \) ATPase activity was measured in the presence of 10 \( \mu \)M CopZ-Cu\(^{2+}\). B and C, Cu\(^{2+}\) transfer from AfCopZ to \( \Delta N, C, \text{CopA} \) wild type (●), S139A/G140A (■) and platform (▲) mutants. Cu\(^{2+}\) (B) and protein (C) contents of the wash and elution fractions are shown. D, representative Western blot using an anti-His\(_6\) and anti-Strep tag antibodies of the peaks corresponding to CopZ (W1) and \( \Delta N, C, \text{CopA} \) proteins (E2) eluted from the Ni\(^2+\)-nitrilotriacetic acid columns. The values are the means ± S.E. (\( n = 3 \)).

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FIGURE 4. Structural model of the C-terminal $Cu^+$ chaperone domain of AfCopZ (CopZ). Cysteines involved in $Cu^+$ binding are highlighted in yellow. Negatively charged residues are indicated in red. These residues were alanine-substituted, creating three different proteins used in this study. A, Ct-CopZ M1 putative Ct-CopZ electronegative exposed surface facing toward the platform of AfCopA (11). B, Ct-CopZ M2 electronegative surface. C, Ct-CopZ M3 electronegative surface.

TABLE 3

| Ct-CopZ-Cu$^+$-dependent ATPase activity kinetic parameters of $\Delta N, C$-CopA and $Cu^+$ binding stoichiometry of A. fulgidus Ct-CopZ wild type and mutants |
|---|
| Ct-CopZ | $V_{max}$ | $K_{m}$ | Stoichiometry$^a$ |
| Wild type | $5.3 \pm 0.35$ | $1.6 \pm 0.42$ | $1.00 \pm 0.07$ |
| M1 | $5.22 \pm 0.80$ | $1.12 \pm 1.5$ | $1.02 \pm 0.05$ |
| M2 | $5.43 \pm 0.50$ | $1.99 \pm 0.67$ | $1.01 \pm 0.11$ |
| M3 | $5.42 \pm 0.42$ | $1.00 \pm 0.35$ | $1.00 \pm 0.07$ |

$^a$ Stoichiometry was estimated as moles of metal/moles of Ct-CopZ. 

Errors for $V_{max}$ and $K_{m}$ are asymptotic standard errors reported by the fitting software Kaleidagraph (Synergy).

of these residues did not affect the ATPase activity when stimulated by free $Cu^+$ in the assay media, nor did this impair $Cu^+$ binding to TM-MBSs (Table 2). It follows that these residues are not required for $Cu^+$ translocation or ATP hydrolysis. Moreover, the mutations do not seem to lead to large unspecific changes in enzyme structure/function. A possible role in the chaperone-mediated $Cu^+$ transfer to the ATPase was subsequently tested (Fig. 6). In this case, none of the Ala-substituted proteins were activated by saturating concentrations of AfCopZ-Cu$^+$; however, the Cys-substituted ATPases were fully active in the presence of AfCopZ-Cu$^+$ (Fig. 6A). Further evidence for the role of these residues was provided by $Cu^+$ transfer determinations, where the Ala-substituted $\Delta N, C$-CopA was unable to take the metal from CopZ-Cu$^+$ (Fig. 6, B and C). These experiments suggest that, at these positions, it is the ability to interact with the metal rather than the side chain charge or size that is relevant for $Cu^+$ transfer.

DISCUSSION

The transfer of $Cu^+$ from soluble chaperones to transmembrane transporters is central to maintain $Cu^+$ homeostasis. As such, this has received significant attention (4, 12, 20, 42). Biochemical studies have helped us to understand how the chaperone delivers $Cu^+$ directly to both TM-MBSs present in $Cu^+$-ATPases (10, 24). This metal transfer is characterized by the transient interaction of the $Cu^+$-loaded chaperone with the ATPase and the unidirectional transfer CopZ-Cu$^+$ + CopA $\rightarrow$ CopZ + CopA-Cu$^+$ rather than the equilibrium CopZ-Cu$^+$ + CopA $\leftrightarrow$ CopZ + CopA-Cu$^+$. However, these studies did not show where or how these proteins interact or how $Cu^+$ is released from the chaperone and taken up by the ATPase. The high resolution structure of LpCopA suggests a region of interaction and residues likely involved in the metal binding by the ATPase (33). Here, we present experimental evidence that supports a model where the $Cu^+$-loaded chaperone interacts with an electropositive platform formed by a kink in the second TM of the ATPase. Subsequently, three invariant ATPase residues participate in the ligand exchange that mobilizes $Cu^+$ from the chaperone to the TM-MBSs. This model is in agreement with previous observations and explains how the metal transfer occurs while the proteins maintain $Cu^+$ sequestered.

The well characterized CopZ-CopA system from A. fulgidus was used to test the proposed model. Homology modeling of these proteins showed that AfCopZ has an electronegative surface that would match the electropositive platform region of the ATPase. In fact, docking of AfCopZ-Cu$^+$ to the platform of $\Delta N, C$-CopA has a negative $\Delta G$ (15). Mutations were designed to remove the electropositive area (PLAT mutant); to alter the kink region, perhaps changing the orientation of the platform helix (S139A/G140A mutant); and to replace single amino acids at the entrance site, either maintaining or removing the metal coordination capability. None of these mutations altered the interaction of $Cu^+$ with CopA or the enzyme turnover kinetics. That is, the metal could access the TM-MBSs independently of the integrity of the entrance site, and the mutated enzymes were able to undergo all conformational transitions apparently at the same rate the background $\Delta N, C$-CopA protein. Therefore, the involved structures are not necessary for chaperone-independent transport or catalytic steps. Alternatively, activation of the CopA ATPase by CopZ-Cu$^+$ was significantly affected by some of these mutations.

Removal of electropositive residues at the platform precluded the activation of the enzyme by the $Cu^+$-loaded chaperone, whereas substitution of the closely located Ser-139/Gly-140 has no significant effect. This suggests that the electropositive charges at the platform are required for CopZ-Cu$^+$ interaction with CopA rather than the structural integrity of the region. Mutation of electronegative residues on the chaperone prevented the activation of the ATPase, whereas substitution of other negatively charged surfaces on the chaperone had no effect. Several lines of evidence further support the idea that electrostatics drive the CopZ-Cu$^+$/CopA interaction through the platform region. The involvement of complementary electrostatic surfaces has been shown for the interaction of $Cu^+$ chaperones with target $Cu$,$Zn$-superoxide dismutases and, most importantly, the regulatory cytoplasmic MBDs in $Cu^+$-ATPases (20, 42, 43). For instance, the yeast Atx1 chaperone has several exposed electropositive residues (Lys-24, Lys-28, Lys-61, and Lys-62) adjacent to the MXXXC $Cu^+$-binding site that may participate in the interaction with the yeast Ccc2 ATPase, because mutation of these residues inter-
fers with Cu\(^{+}\) delivery to Ccc2 N-MBDs (27, 28). Polar residues on the surfaces of Cu\(^{+}\) chaperones are not conserved (19), nor are the amino acids in the platform region. Nevertheless, complementing polarities are likely in these proteins because they would contribute to the specificity of the interaction. Lastly, we might consider that the Cu\(^{+}\) positive charge is required for the interaction, because the apo chaperone cannot compete for the docking site with the holo-form, nor can it extract Cu\(^{+}\) bound to TM-MBS (24).

Substitution of the invariant residues likely to constitute an entrance site in the ATPases prevented CopZ-bound Cu\(^{+}\) to access TM-MBSs. Significantly, replacement of these amino acids by metal ligating Cys did not impair the enzyme function. It follows logically that the CopZ-Cu\(^{+}\)-CopA interaction leads to positioning the exposed Cu\(^{+}\)-binding site (CXXC) in CopZ toward a Cu\(^{+}\) entry site in the ATPase and might even reduce the affinity of CopZ for the metal, allowing release into the proximity of TM-MBSs. However, a ligand exchange among both these proteins is apparent. This would be reminiscent of alkali metal transporters stripping ions from the hydrating water, replacing these by electronegative side chains and backbone carbonyls. In the case of transition metals, water is unlikely a part of this process, considering the high Cu\(^{+}\) affinity of CopZ and the fact that Cu\(^{+}\) should remain protein-bound to prevent release into the media. Moreover, ligand exchange is the mechanism for Cu\(^{+}\) transfer among chaperone and MBDs (20, 42, 43). The LpCopA structure and our results suggest that Cu\(^{+}\) release from the chaperone requires the coordination of the metal by three invariant residues: Met, Glu, and Asp. It is not clear at this time whether they simultaneously interact with the ion or whether they constitute a ligand chain carrying the ion to TM-MBSs. However, it should be kept in mind that the LpCopA structure describes the enzyme in an E2 conformation (33). That is, TMs and TM-MBSs are not compatible with cytoplasmic metal binding. It cannot then be assumed that the entrance site formed by the three invariant residues is in a conformation suitable for its function when the enzyme is in the E1 form (15, 16). In any case, the data suggest that the metal binding to the entrance is transient, because a metal bound to these residues cannot be detected. Interestingly, the metal transfer is viable.

![Figure 5. ΔN.C-CopA interaction with the electronegative surface of Ct-CopZ.](image-url)

A, ΔN.C-CopA-Cu\(^{+}\)-dependent ATPase activity. Wild type and mutated Ct-CopZ-dependent ATPase activity was measured in the presence of 10 μM Ct-CopZ-Cu\(^{+}\). B and C, Cu\(^{+}\) transfer from wild type (●) and mutated, M1 (○), M2 (○), and M3 (■) Ct-CopZ to ΔN.C-CopA. Cu\(^{+}\) (B) and protein (C) contents of the wash and elution fractions are shown. D, representative Western blot using an anti-His\(_{6}\) and anti-Strep tag antibodies of the peaks corresponding to CopZ (W1) and ΔN.C-CopA proteins (E2) eluted from the Ni\(^{2+}\)-nitrilotriacetic acid columns. The values are the means ± S.E. (n = 3).
even if the carboxyl side chains are replaced by Cys. However, no cysteines are present in the hundreds of available Cu+/H11001-ATPase sequences, suggesting that Cys in this exposed position might be susceptible to redox modifications deleterious to ATPase function.

In summary, the data provide evidence supporting a plausible mechanism for Cu+ access to transmembrane transport sites. This chaperone-transporter interaction and ligand exchange-mediated metal delivery is likely to be mirrored at the transporter exit site to enable metal release. Moreover, similar mechanisms might be in place in other transition metal transporters.

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