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Chaperone-mediated Cu^+ Delivery to Cu^+ Transport ATPases

REQUIREMENT OF NUCLEOTIDE BINDING[*][S]

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Cu^+-ATPases drive the efflux of Cu^+ from the cell cytoplasm. During their catalytic/transport cycle, cytoplasmic Cu^+-chaperones deliver the metal to the two transmembrane metal-binding sites (TM-MBSs) responsible for Cu^+ translocation. Here, using *Archaeoglobus fulgidus* Cu^+-ATPase CopA and the C-terminal Cu^+-chaperone domain of CopZ (Ct-CopZ), we describe the mechanism of Cu^+ transfer to both TM-MBSs. In absence of other ligands, Ct-CopZ transfers Cu^+ to wild-type CopA and to various CopA constructs lacking or having mutated cytoplasmic metal-binding domains, in a fashion consistent with occupancy of a single TM-MBS. Similar experiments performed in the presence of 2.5 mM ADP-Mg^{2+}, stabilizing an *E*1·ADP, lead to full occupancy of both TM-MBSs. In both cases, the transfer is largely stoichiometric, *i.e.* equimolar amounts of Ct-CopZ·Cu^+ saturated the TM-MBSs. Experiments performed with CopA mutants lacking either TM-MBS showed that both sites are loaded independently, and nucleotide binding does not affect their availability. The nucleotide-induced *E*2→*E*1 transition is structurally characterized by a large displacement of the A and N domains opening the cytoplasmic region of P-type ATPases. Then, it is apparent that, whereas the first Cu^+-chaperone can bind an ATPase form available in the absence of ligands, the second requires the *E*1·nucleotide intermediary to interact and deliver the metal. Interestingly, independent of TM-MBS Cu^+ loading, nucleotide binding also prevents the regulatory interaction of the N-terminal cytoplasmic metal-binding domain with the nucleotide binding domain.

Copper is an essential micronutrient involved in many critical processes (1, 2). However, it can also cause cellular damage due to its ability to catalyze the production of free radicals. To maintain the cell integrity while ensuring an adequate supply of copper, organisms have developed a complex molecular machinery to mobilize the metal. This involves copper-dependent transcription factors, chelators that sequester the excess metal, Cu^+-chaperones that shuttle it to target proteins, and transporters that facilitate Cu^+ translocation across the membranes (3–8). Although the presence of chelators and Cu^+-chaperones ensures the absence of free copper in the cell, it also creates the need of singular transmembrane transporters. These, although having structures similar to alkali metal carriers and pumps, should possess distinct transport mechanisms that deal with protein-metal and protein-protein affinity constants rather than metal concentration gradients across membranes (4, 6, 9, 10). Consequently, metal transport characteristics such as rate, specificity, energy requirement, etc., should be evaluated in light of these alternative paradigms.

Cu^+-ATPases are responsible for cytoplasmic Cu^+ efflux. They are members of the P-type ATPase family of ion transporters, which also includes the well characterized Ca^{2+}-ATPases, Na^+/K^+-ATPases and H^+-ATPases, among others (11–13). As all P-type ATPases, Cu^+-ATPases couple ion (Cu^+) transport to the hydrolysis of ATP following the essential elements of a classic Albers-Post cycle (4, 14). Briefly, cytoplasmic Cu^+ binding to two transmembrane metal-binding sites (TM-MBSs)[2] is coupled to ATP hydrolysis and enzyme phosphorylation ($E1P(Cu^+)_2$). Subsequently, the enzyme undergoes a conformational change (to *E*2P) leading to TM-MBSs opening to the vesicular/extracellular compartment with the consequent metal release. Enzyme dephosphorylation allows the return to the *E*1 form with TM-MBSs facing the cytoplasm. It is relevant that the *E*2→*E*1 transition is accelerated by ATP (or ADP) acting with low affinity; *i.e.* a modulatory mode (4, 15–19).

Cu^+-ATPases consist of eight transmembrane helices, two large cytosolic loops comprising the actuator (A) and the ATP-binding domain (ATP-BD), which includes the phosphorylation (P) and nucleotide (N) domains, and cytoplasmic metal-binding domains (MBDs) in the N terminus (4, 11, 20–24) (see Fig. 1). The number of N-MBDs varies from one in bacteria and archaea to six in higher eukaryote ATPases. Cytoplasmic MBDs have a conserved $\beta\alpha\beta\beta\alpha\beta$ fold and an invariant C*XX*C metal-binding sequence similar to the well described Cu^+-chaperones, Atox1, Atx1, and CopZ (25–27). N-MBDs have a regulatory role controlling the transporter turnover rate (28–30). In addition, they are involved in the important sorting of Cu^+-ATPases observed in eukaryotes (14). Lutsenko and collaborators have shown that a protein construct containing the six N-MBDs present in the human ATP7B Cu^+-ATPase interacts with the ATP-BD in a Cu^+-dependent manner (31). Recently obtained structures of *Archaeoglobus fulgidus* CopA show the

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3 and Table S1.

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[2] The abbreviations used are: TM-MBS, transmembrane metal-binding site; BCA, bicinchoninic acid; ΔN,C-CopA, CopA lacking both MBDs; C_2-CopA, CopA where MBDs have been mutated; $C_2^{N\text{-}MBD}$-CopA, CopA where Cys in TM-MBSs and C-MBD have been mutated; C_0-CopA, Cys-less CopA; A-domain, actuator domain; ATP-BD, ATP-binding domain; P-domain, phosphorylation domain; N-domain, nucleotide domain; MBD, metal-binding domain; BP fraction, bound protein fraction; UP fraction, unbound protein fraction.

physical proximity between its single N-MBD, the A-domain, and the ATP-BD (24). These two observations could provide a structural basis for the regulatory role of cytoplasmic MBDs via Cu^+-dependent domain-domain interactions (28–32).

Transmembrane helices H6, H7, and H8 contain six conserved amino acids that form two metal-binding sites responsible for transmembrane transport: TM-MBS-I (two Cys in H6 and a Tyr in H7) and TM-MBS-II (Asn in H7, Ser and Met in H8) (see Fig. 1) (9). Both sites coordinate Cu^+ in a trigonal planar geometry. Mutation of any of these residues removes binding to the corresponding site and precludes Cu^+-dependent partial reactions (33). Cytoplasmic Cu^+ accesses the TM-MBSs bound to specific Cu^+-chaperones (34). Consequently, a specific transient interaction between the metal-loaded chaperone and the transmembrane ATPase by which the metal is delivered to TM-MBS can be predicted. This interaction and the associated Cu^+ transfer are independent of Cu^+ binding to N-MBDs, because these are not required for ATPase activation by chaperone bound Cu^+. Both TM-MBSs bind Cu^+ with high affinity ($K_a^{I} = 1.12 \pm 0.25\ fM^{-1}$, and $K_a^{II} = 1.3 \pm 0.22\ fM^{-1}$) preventing its backward release to cytoplasm (9). Thus, saturation of both sites is easily achieved during equilibrium Cu^+ binding assays performed with purified micellar proteins and "free" Cu^+ ion; *i.e.* in the absence of metal chaperones. However, chaperone-mediated Cu^+ transfer to the TM-MBS under equilibrium conditions leads to occupancy of a single site (34). If, as previously shown (33), metal binding to both TM-MBS is required for enzyme turnover, how is then the second TM-MBS loaded? High resolution structures of the sarcoplasmic reticulum Ca^{2+}-ATPase have shown the large movements and coordinated interaction of cytoplasmic A-domain and ATP-BD during the catalytic cycle in P-type ATPases. These catalytic events driven by nucleotide binding, phosphorylation and dephosphorylation, are critical for metal binding, transmembrane occlusion, and translocation (35, 36). Experiments using limited proteolysis of *Thermotoga maritima* Cu^+-ATPase suggest that similar large domain rearrangements also occur in heavy metal ATPases during transport (37). Could the ligand-driven conformational transitions be required for chaperone-mediated Cu^+ loading of transport sites?

Employing the simple and well characterized *A. fulgidus* Cu^+-ATPase CopA, we have further explored the Cu^+ transfer into TM-MBSs by the corresponding chaperone, CopZ. We have observed that the equilibria for either TM-MBS (Ct-CopZ·Cu^+ + TM-MBDs → Ct-CopZ + TM-MBDs·Cu^+) are fully displaced toward saturation of CopA TM-MBSs. However, nucleotide binding to the ATP-BD is required for metal occupancy of the second transport site. This not only affects the CopZ-mediated Cu^+ transfer to CopA but also disrupts the independent interaction of the regulatory N-MBD with ATP-BD.

EXPERIMENTAL PROCEDURES

cDNA Cloning, Protein Expression, and Purification—The *A. fulgidus* CopA and CopZ constructs used in this work are listed in the supplemental Table S1. cDNA coding for the A-domain of CopA was amplified from the CopA cDNA by PCR using primers 5′-ATGGGGGAGGCCATAAAGAAGCTCGTA-3′ and 5′-GCCCATCGCGTCCTCGACCAGCTT-3′. This DNA was cloned into pBAD/TOPO vector, which introduces a C-terminal hexahistidine tag suitable for Ni^{2+} affinity purification, and transformed into *Escherichia coli* Top10 cells. S715A $C_2^{N\text{-}MBD}$-CopA was the result of the ligation of the DNA fragments obtained from the HindIII and BsmI digestion of S715A C_2-CopA and Wt-CopA previously cloned in pCRT7/NT-TOPO vector (9, 15). This construct was subsequently transformed in B127(DE3)pLysS *E. coli* cells (Invitrogen). Expression and purification of membrane and soluble proteins were carried out as described (9, 15, 22, 38). Protein determinations were performed in accordance with Bradford (39).

Cu^+ Transfer Assay—Cu^+ transfers from Strep-tagged Ct-CopZ to different His-tagged CopA constructs were performed as previously described (34). Cu^+-loaded Ct-CopZ was obtained by incubating equimolar amounts of Ct-CopZ and $CuSO_4$ in a buffer containing 25 mM HEPES, pH 8.0, 500 mM NaCl, 10 mM ascorbic acid, and 0.01% *n*-dodecyl-β-D-maltopyranoside, 0.01% asolectin (Buffer T) at room temperature for 5 min with gentle agitation. No free Cu^+ was detected after passing the Cu^+-loaded Ct-CopZ through a Sephadex G-25 column (Sigma) and measuring Cu^+ levels with the bicinchoninic acid (BCA) assay (40). Except when indicated, Cu^+ transfer assays were carried out by incubating 5 μM of indicated CopA constructs with concentrations of Ct-CopZ·Cu^+ corresponding to 1.5 times the number of Cu^+-binding sites available in the CopA construct; for example, 15 μM Ct-CopZ·Cu^+ in the case of C_2-CopA (which has two Cu^+-binding sites) or 7.5 μM Ct-CopZ·Cu^+ for C_0-CopA (which has a single Cu^+ site remaining) (9). Cu^+ transfer was performed in buffer T. 2.5 mM ADP-$MgCl_2$ was included in the transfer assay media when the effect of nucleotide was tested. Following incubation at room temperature for 5 min, 150 μl of Strep-Tactin resin (IBA) pre-equilibrated in buffer T was added to bind Ct-CopZ. Unbound protein was separated by centrifugation at 14,000 rpm for 2 min. Resin-bound proteins were washed with 1 ml of buffer T, followed by elution with 0.5 ml of 2.5 mM desthiobiotin in buffer T. Protein and Cu^+ concentration in the unbound and bound protein fractions were determined. SDS-PAGE (15% acrylamide gel) was performed to verify that only CopA-derived proteins were present in the unbound protein fraction and that only Ct-CopZ was present in the elution fraction (supplemental Fig. S1). Controls were performed where Cu^+-loaded Ct-CopZ or CopA constructs were individually subjected to the same procedures; *i.e.* lacking the partner protein. Previously reported results showed that during these assays no significant amount of Cu^+ is released from CopZ (34).

Domain-Domain Co-purification Assays—Interactions among cytoplasmic domains of CopA were studied by assessing the co-purification of isolated domains by batch affinity chromatography. 20 μM His-tagged ATP-BD or A-domain and 40 μM Strep-tagged N-MBD were incubated in a buffer containing 25 mM Tris-HCl, pH 8.0, 250 mM NaCl, 50 mM sucrose, and 5 mM dithiothreitol (Buffer I) for 10 min at room temperature with gentle agitation. The effects of Cu^+ and nucleotides on domain-domain interaction were investigated by using Cu^+-loaded N-MBD or including 5 mM ADP-$MgCl_2$ in the assay media. Cu^+-loaded N-MBD was obtained by incubating the

protein with a five molar excess of $CuSO_4$ in 25 mM Tris-HCl, pH 7.5, 10 mM ascorbic acid for 10 min at room temperature with gentle agitation. Unbound Cu^+ was removed by passing through a Sephadex G-25 column (Sigma). Efficient Cu^+ loading was verified by measuring Cu^+ content of the eluted protein using the BCA assay (40). The Cu^+-loaded N-MBDs was used immediately after removing unbound Cu^+ to minimize Cu^+ dissociation. 200 μM bathocuproindesidulfonic acid was added to the reaction when Cu^+-free conditions were required. Samples were incubated with 20 μl of Ni^{2+}-nitrilotriacetic acid resin (Qiagen) for 10 min at room temperature and centrifuged at 14,000 rpm for 5 min to collect the unbound proteins in the supernatant (UP fraction). The proteins bound to the resin were washed with 200 μl of 5 mM imidazole in Buffer I and 200 μl of 20 mM imidazole in Buffer I, followed by elution with 50 μl of 150 mM imidazole in Buffer I (BP fraction). Protein content in the UP and BP fractions was analyzed by SDS-PAGE using 15% acrylamide gels and visualized by Coomassie Brilliant Blue staining (41). Controls were performed where each protein domain was individually subjected to the same procedures; *i.e.* lacking the interacting partner.

RESULTS

Effect of Nucleotide Binding on the Cu^+ Transfer from CopZ·Cu^+ to CopA—Activation of Cu^+-ATPases by Cu^+-chaperones is independent of the presence or functionality of the N-MBD (or C-MBD) (34). Thus, chaperones deliver Cu^+ directly to the TM-MBDs of Cu^+-ATPases. However, Cu^+ transfer experiments performed under equilibrium conditions using excess of Cu^+-chaperone to Cu^+-ATPase lacking MBDs (CopZ·Cu^+:ΔN,C-CopA, 3:1 molar ratio) showed transfer to and occupancy of an apparently single Cu^+ site, albeit there are two TM-MBSs (9, 34). This observation could be explained by the requirement of either larger excess of chaperone·Cu^+ or functional N-MBDs for full occupancy of transport sites. Alternatively, because the metal transfer occurs through protein-protein interaction (34), the need of a specific enzyme conformation able to open both TM-MBSs to chaperone·Cu^+ can be postulated. Testing these hypotheses, we measured Cu^+ transfer from the Cu^+-loaded C terminus domain of *A. fulgidus* CopZ to various *A. fulgidus* CopA constructs. Assays were performed under equilibrium (non-turnover) conditions using soluble (micellar) forms of CopA. Ct-CopZ contains the classic Cu^+-chaperone structure but lacks the ferredoxin-like N-terminal domain present in *A. fulgidus* CopZ (38). CopA proteins included Wt-CopA, CopA lacking one or both MBDs (ΔN,C-CopA and ΔN-CopA), and CopA containing mutated MBDs unable to bind Cu^+ (C_2-CopA). Previously reported control assays showed no appreciable release of free Cu^+ during this type of experiment (34). Table 1 shows that, in agreement with our previous observations, in the absence of other enzyme ligand Ct-CopZ·Cu^+ transferred one Cu^+ to TM-MBSs present in ΔN,C-CopA and C_2-CopA. Experiments performed with up to five times molar excess of Ct-CopZ·Cu^+ yielded identical results (not shown). Further supporting the single occupancy of TM-MBS under similar conditions, ΔN-CopA accepted two Cu^+ from Ct-CopZ·Cu^+, one in the C-MBD and the other in one of the two available TM-MBSs. Wt-CopA showed only slightly higher values (Table 1). However, analysis of this result is complicated by the partial transfer (not stoichiometric) of Cu^+ from Ct-CopZ to N-MBD. We have reported that equilibrium exchange experiments performed with six times excess CopZ·Cu^+ to N-MBD yielded only 35% of N-MBD·Cu^+ (38). Control experiments performed with a full-length CopA construct where the only remaining Cu^+ site was at the N-MBD (S715A $C_2^{N\text{-}MBD}$-CopA) showed a similar equilibrium with only 24–34% Cu^+ loading of N-MBD in the presence of 1.5 excess Ct-CopZ·Cu^+ (Table 1). On the contrary, CopZ·Cu^+ fully transfers the metal to C-MBD (34). Thus, it could be assumed that in the Wt-CopA one Cu^+ occupies the C-MBD, a second is in a TM-MBS, and the N-MBD is partially occupied. Although these data indicated that the MBDs are indeed not necessary for Ct-CopZ·Cu^+ delivery of Cu^+ to CopA TM-MBDs, they do not provide an explanation for the apparent loading of a single TM-MBS. We realize that the described results could also be explained by 50% occupancy of each TM-MBS. However, experiments described below, using mutants where a single TM-MBS is available, show that this is not the case.

TABLE 1
Cu^+ transfer to CopA by Ct-CopZ

Construct	Cu^+ ions/CopA molecule[a]	
	No nucleotide	2.5 mM ADP-$MgCl_2$
ΔN,C-CopA	0.8 ± 0.1[b]	1.8 ± 0.2
C_2-CopA	1.1 ± 0.2	1.9 ± 0.1
ΔN-CopA	1.9 ± 0.2	2.9 ± 0.3
Wt-CopA	2.2 ± 0.1	3.1 ± 0.1
S715A $C_2^{N\text{-}MBD}$-CopA	0.2 ± 0.2	0.3 ± 0.1
N683A C_0-CopA	0.1 ± 0.0	0.0 ± 0.0
ΔN-CopA/Ct-CopZ	1:2 ratio	1:3 ratio
	2.0 ± 0.1	2.8 ± 0.1

[a] Values are the mean ± S.E. (n = 3).
[b] Datum was published in Ref. 34.

Loading of TM-MBDs sites by "free" Cu^+ has shown that both sites accept the metal in the absence of any ligand stabilizing a particular conformation (9). Despite this, a certain possibility was that the interaction of the second Ct-CopZ·Cu^+ with CopA requires a different enzyme conformation. Testing this hypothesis, Cu^+ transfer experiments were performed in the presence of 2.5 mM ADP-Mg^{2+}. This drives the enzyme into an *E*1 conformation, but phosphorylation followed by Cu^+ release cannot occur. Under this condition, full occupancy of available TM-MBSs was obtained for all constructs (Table 1). Ct-CopZ-mediated Cu^+ transfer to a mutant CopA lacking all four Cu^+-binding sites (N683A C_0-CopA) was measured to verify the absence of Cu^+ transfer to an adventitious site. As expected, no Cu^+ was transferred to this mutant (Table 1). Thus, these observations pointed out that the availability of both TM-MBSs to accept Cu^+ from the chaperone requires a conformational change driven by binding of nucleotide (ATP under turnover conditions).

Unidirectional Cu^+ Transfer from Ct-CopZ to CopA TM-MBSs—Interestingly, these experiments revealed additional mechanistic information. Because these assays were performed with little excess of Ct-CopZ·Cu^+ over the available Cu^+ sites in CopA proteins (1.5:1 molar ratio for Cu^+ sites), the data suggest full Cu^+ transfer from Ct-CopZ·Cu^+ to both TM-MBSs, rather than an exchange where a measurable equi-

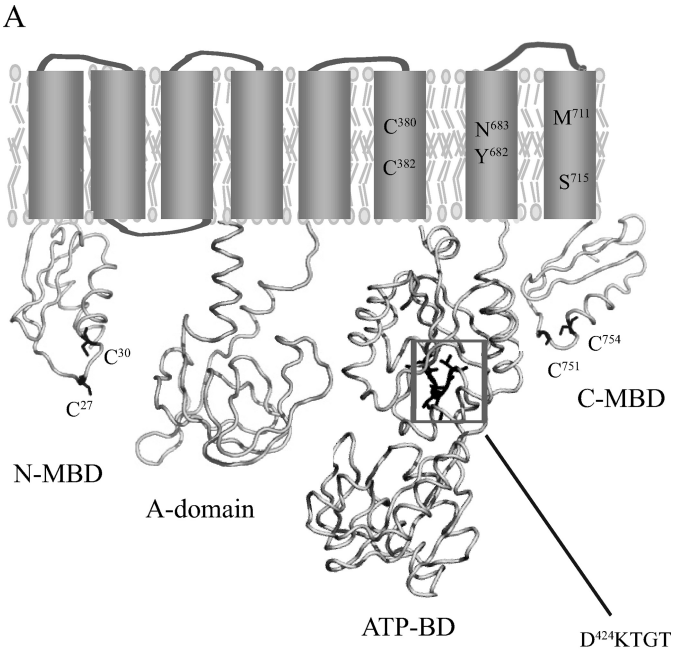

FIGURE 1. **Scheme of CopA structure (*A*) and transmembrane Cu^+-binding sites (*B*).** The topological scheme of CopA includes the A-domain (PDB 2HC8) and ATP-BD (PDB 2B8E) structures. N-MBD and C-MBD structures are homology models based on the fourth N-MBD of the Menkes protein (PDB 1AW0) and in *Bacillus subtilis* CopZ (PDB 1K0V), respectively. Constructs ΔC-CopA and ΔN,C-CopA lack one or both MBD domains. C_2-CopA has Ala substitutions in the Cys present in N-MBD and C-MBD. $C_2^{N\text{-}MBD}$-CopA has Ala substitutions in the Cys present in TM-MBSs and C-MBD. C_0-CopA has all Cys in the molecule replaced by Ala. Amino acids involved in the Cu^+ coordination by the TM-MBS are indicated in *B*.

librium is established. This stoichiometric delivery was further verified testing Cu^+ transfer from Ct-CopZ·Cu^+ to ΔN-CopA (three Cu^+ sites: two in the TM-MBS and one in C-MBD) in a molar ratio 2:1 (no nucleotide) or 3:1 (in the presence of nucleotide). Indeed, under this condition the chaperone fully handed over the Cu^+ to the transport sites (Table 1).

Ct-CopZ Transfers Cu^+ to Either TM-MBS—The data showed in Table 1 suggest that in the absence of nucleotide either a CopZ interaction site is not accessible or, alternatively, one of the TM-MBSs is unavailable. Either possibility suggests that TM-MBSs can be loaded sequentially or independently. TM-MBSs in *A. fulgidus* CopA are formed by Cys-380, Cys-382, and Tyr-682 (Site I) and Asn-683, Met-711, and Ser-715 (Site II) (Fig. 1) (9). Mutation of any given residue results in the loss of the Cu^+-binding ability in the corresponding TM-MBS. To identify whether in the absence of nucleotide Ct-CopZ delivers Cu^+ to a particular TM-MBS, Cu^+ transfer assays were carried

TABLE 2
Cu^+ transfer to CopA TM-MBS mutants by Ct-CopZ

Construct	Cu^+ ions/CopA molecule[a]	
	No nucleotide	2.5 mM ADP-$MgCl_2$
C_0-CopA	1.1 ± 0.0	1.1 ± 0.0
M711A C_2-CopA	1.1 ± 0.3	1.2 ± 0.1
C382A C_2-CopA	1.2 ± 0.2	1.2 ± 0.3
Y682A C_0-CopA	1.1 ± 0.1	1.2 ± 0.3
S715A C_2-CopA	1.0 ± 0.0	1.1 ± 0.3

[a] Values are the mean ± S.E. ($n = 3$).

out between Ct-CopZ·Cu^+ and M711A C_2-CopA (only Site I is functional) or C_0-CopA (only Site II is functional). Surprisingly, both mutants were able to accept Cu^+ from the chaperone (Table 2). However, it might be argued that some of the remaining amino acids in these constructs could be involved in facilitating the transfer of Cu^+ from the chaperone to the other site. Following this model, Cu^+ transfer would occur as a ligand exchange between the Cys of Ct-CopZ and one amino acid from a specific TM-MBS, in a fashion similar to that proposed for the transfer between chaperone and MBDs (42, 43). As a result, Cu^+ delivery by the chaperone could still be sequential, and our observations would be the result of not having mutated the Cu^+-accepting amino acid. To address this issue, Cu^+ transfer experiments were carried out between Ct-CopZ·Cu^+ and C382A C_2-CopA, Y682A C_0-CopA, or S715A C_2-CopA, which lack the cytosolic-oriented Cu^+ coordinating residues in Site I or II (Cys-382, Tyr-682, and Ser-715) (Fig. 1). As indicated in Table 2, all of these CopA mutants were able to accept one Cu^+ ion from Ct-CopZ. Moreover, the presence of nucleotide had no effect in the loading of any of these mutants. Toward explaining this result, it could be proposed that mutations in the first loading site might "lock" CopA in a form similar to *E*1·ADP. However, biochemical characterization of these mutants has shown that they are in an *E*1↔2 equilibrium similar to the wild-type protein, as evaluated by P_i phosphorylation (33). Consequently, it is apparent that removal of any of the Cu^+-ligating amino acid side chains did not affect the ability of Ct-CopZ to deliver Cu^+ to the remaining TM-MBS, which would occur in an independent, non-sequential manner.

Interactions between CopA Cytoplasmic Domains—Placing CopA in a nucleotide-bound form appears to be required for fully loading of the metal transport sites by Ct-CopZ·Cu^+. Crystallographic studies of Ca^{2+}-ATPases indicated a significant rearrangement, particularly of cytoplasmic regions upon nucleotide binding (16, 35, 36). The structure of CopA in the absence of ligands obtained by cryoelectron microscopy suggests the proximity of N-MBD to both ATP-BD and A-domain (24). Then, does the nucleotide-driven conformational change affect other aspects of the enzyme function, such as the interaction with regulatory domains (31)? *A. fulgidus* CopA cytoplasmic domains (N-MBD, A-domain, and ATP-BD) can be expressed in isolated forms. These maintain their structures and ligand (Cu^+ or nucleotides)-binding capabilities (9, 21, 22). The putative interactions between these domains were examined by determining their co-purification by batch affinity chromatography where domain pairs carried alternative tags, either $(His)_6$ or Strep tags (supplemental Table S1). Co-purification experiments

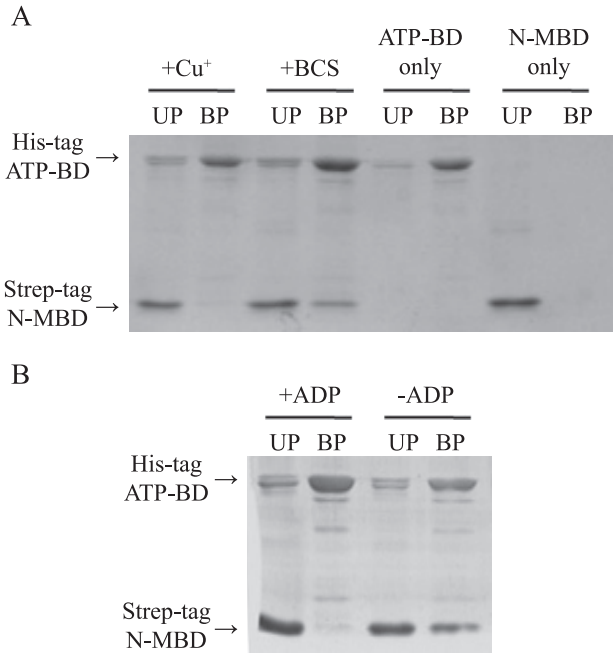

FIGURE 2. **Interaction between N-MBD and ATP-BD.** *A*, SDS-PAGE of a representative co-purification assay between ATP-BD and N-MBD or Cu^+-loaded N-MBD. *B*, SDS-PAGE of a representative co-purification assay between N-MBD and ATP-BD in the absence and in the presence of 5 mM ADP-Mg^{2+}. 40% of unbound protein (*UP*) or 40% of the bound protein (*BP*) fractions were loaded in each *lane*.

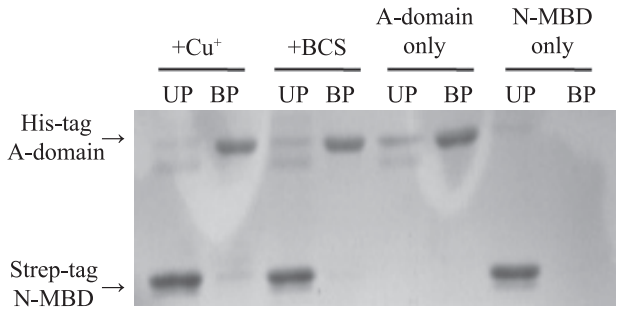

FIGURE 3. **Interaction between N-MBD and A-domain.** SDS-PAGE of a representative co-purification assay between A-domain and N-MBD or Cu^+-loaded N-MBD. 40% of unbound protein (*UP*) or 40% of the bound protein (*BP*) fractions were loaded in each *lane*.

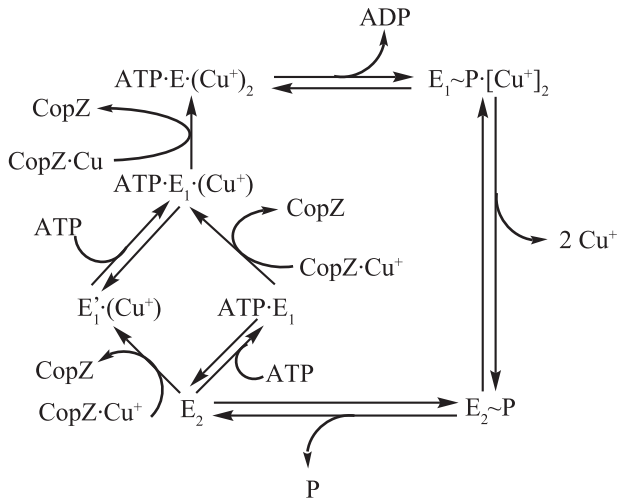

FIGURE 4. **Proposed catalytic and transport cycle of Cu^+-ATPases.**

indicated that the N-MBD does interact with ATP-BD (Fig. 2). Fig. 2*A* shows that, whereas Cu^+-loaded N-MBD did not form a stable interaction with the ATP-BD, the Cu^+-free Strep-N-MBD upon incubation with His-ATP-BD remained associated to Ni^{2+}-resin. Control experiments showed that in the absence of ATP-BD, Strep-N-MBD did not bind the Ni^{2+}-resin. Alternatively, assays were performed by co-purifying the N-MBD·ATP-BD complex using Streptactin resin yielded equivalent results (supplemental Fig. S2*A*). Similarly, this interaction and its prevention by the presence of metal were observed in cross-linking experiments (supplemental Fig. S2*B*). As previously proposed by Tsivkovskii *et al.* (31), it appears that the binding of Cu^+ to the N-MBD prevents the interaction with the ATP-BD. Moreover, Fig. 2*B* shows that the presence of saturating ADP-Mg^{2+} also prevented N-MBD·ATP-BD co-purification. As in previous experiments, ADP was used instead of ATP to avoid any slow ATP hydrolysis driven by the ATP-BD. These observations indicate that conformational changes driven by binding of substrate (nucleotide to ATP-BD) or regulatory ligand (Cu^+ to N-MBD) affect the interaction among these domains. The association of N-MBD with ATP-BD is specific, because control experiments testing the interaction of N-MBD with the ATP-BD from the Na^+/K^+-ATPase showed no co-purification (supplemental Fig. S3). The probable association of N-MBD with A-domain has been suggested by structural (24) and biochemical studies (37). However, no interaction among these domains was observed in co-purification experiments performed in the absence or presence of Cu^+ (Fig. 3). Cross-linking experiments among these domains were prevented by high homodomain interactions under all tested experimental conditions (not shown).

DISCUSSION

Recent observations have indicated that Cu^+-chaperones directly transfer the transported Cu^+ to the corresponding ATPase TM-MBSs via protein-protein interactions, guaranteeing that the metal is at no time released into the cytoplasm (34). Experiments testing equilibrium binding of free Cu^+ to the CopA have shown the presence of two TM-MBSs with femtomolar affinities for the metal (34). In agreement with well described mechanistic characteristics of P-type ATPases, both sites need to be occupied for catalytic enzyme phosphorylation and subsequent transport (35). However, chaperone-mediated metal transfer to the ATPase TM-MBSs under equilibrium conditions leads to loading of a single TM-MBS (34). The data reported here explain previous findings and support a mechanism of Cu^+ transfer consistent with the transport requirements (Fig. 4). In addition, they reveal distinct properties of Cu^+-ATPases directly impacting metal homeostasis.

Conformational Requirements for Chaperone-mediated Cu^+ Transfer to the Cu^+-ATPase TM-MBS—Our studies allow a more detailed mechanistic description of the catalytic/transport cycle of Cu^+-ATPases (Fig. 4). This scheme takes into account the presence of two TM-MBSs (9), that apo-CopZ does not "compete" with CopZ·Cu^+ for interaction sites during acti-

vation of CopA ATPase (34) and the unidirectional stoichiometric Cu^+ transfer to TM-MBS. Consequently, CopZ-mediated Cu^+ loading of TM-MBS is represented by *single arrows* in the scheme of CopA catalytic cycle.

Experiments reported here show that, under equilibrium conditions and in the absence of other enzyme ligands, Ct-CopZ delivers a single Cu^+ to Wt-CopA TM-MBSs. This is, in the absence of nucleotide binding, the first Cu^+ places the enzyme in a conformation (*E*1′) apparently unable to interact with CopZ·Cu^+. However, the *E*1·ADP form is able to receive two Cu^+ ions in the TM-MBSs from CopZ·Cu^+. Similar results were obtained with CopA constructs carrying mutated MBDs unable to bind metals or lacking MBDs. These data re-emphasize the idea that the MBDs are not required for loading of TM-MBSs (34). Classic studies of ion binding to P_{II}-type ATPases, such as Na^+/K^+-ATPase and Ca^{2+}-ATPases, show that these proteins can independently bind either the ion (Ca^{2+} for instance) or nucleotide (ATP) stabilizing the *E*1 form of the enzyme (18, 44). That is, no ATP binding is required for fully loading the cytoplasmic facing ion sites. It is assumed that even the *E*2 form of the enzymes upon dephosphorylation opens the cytoplasmic ion gates, releases the transported ions (H^+ in the case of the Ca^{2+} ATPase), and can bind the counter-ion (Ca^{2+}), albeit with low affinity (17, 36, 45). Under turnover conditions, the *E*2→*E*1 transition is accelerated by nucleotides binding with low (submillimolar) affinity in a "modulatory mode" leading to cytoplasmic facing high affinity ion binding sites (18, 44). In the case of Cu^+-ATPases, although binding of free Cu^+ appears to follow characteristics similar to that of alkali ion binding to P_{II}-type ATPases, the physiological Cu^+ transfer via protein-protein interactions is clearly distinct. It can be hypothesized that the interaction is different whether the enzyme is in the *E*2↔*E*1 equilibrium (absence of ligands) or the *E*1·ATP form (*E*1·ADP in our experiments). The dramatic conformational change in the cytoplasmic domains during the *E*2→*E*1 transition is demonstrated by the structures of Ca^{2+}-ATPase (17, 36, 45). In *E*2, the cytoplasmic A, P, and N domains are in close proximity; whereas in *E*1 the A and N domains undergo significant displacements. This change is associated with transmembrane fragments rearrangement and ATP binding with high affinity. The role of nucleotide accelerating the *E*2→*E*1 transition is supported by reported structures showing the nucleotide binding to the *E*2 form (16). Because the nucleotide is not required for free Cu^+ binding to CopA TM-MBS, we postulate that nucleotide binding does not change the availability of Site I or II, but is required for the interaction of the second CopZ·Cu^+ (Fig. 4).

The alternative binding of nucleotide or Cu^+-chaperone to the enzyme is apparent from our *in vitro* experiments. Whether ATP or the Cu^+-chaperone binds the enzyme first when both are present in the system, is at this point ambiguous and is represented by a branched pathway in Fig. 4. On one side, CopZ·Cu^+ drives a much higher ATPase turnover than Cu^+ (34). One possible explanation is that the chaperone binds first, accelerating the rate-limiting *E*2→*E*1 transition. In this case, we do not suggest that the chaperone delivers Cu^+ to *E*2. The *E*2 form cannot bind Cu^+ (9). Rather, we take into account the possibility that the chaperone-Cu^+ might interact with *E*2, driving a conformational change to *E*1′, and then Cu^+ is transferred. However, if this were the case, a lower ATP K_m for ATPase activation should be evident when CopZ·Cu^+ is driving the ATPase activity. We have not observed this effect. This is, ATP activates the enzyme with low affinity (K_m 0.5 mM) even in the presence of CopZ·Cu^+.[3] Although this last result suggests the initial binding of ATP, certainly, further analyses are required to understand the interaction of chaperone-Cu^+ with Cu^+-ATPases.

Regulatory Interaction between N-MBD and ATP-BD—The role of N-MBDs has been the focus of much attention. Our data show that the single N-MBD in CopA interacts with the ATP-BD. This brings additional experimental support to the CopA structure proposed by Wu *et al.* (24). Cu^+ binding to N-MBD prevents this interaction in a manner similar to that previously described for the six N-MBDs present in the human ATP7B with its corresponding ATP-BD (31). However, our data hint at a specific role of the N-MBD proximal to the membrane in the proposed control of turnover kinetics observed in all Cu^+-ATPases. In relation with the described effect of low affinity nucleotide binding on Cu^+ delivery to TM-MBSs, it is interesting that millimolar ADP also prevents the N-MBD·ATP-BD interaction. However, this effect does not exclude the regulatory role of Cu^+ binding to N-MBD, because this is observed in the ATPase activity of full-length proteins when measured at saturating nucleotide concentrations (29).

Studies using limited proteolysis of *Thermotoga maritima* CopA have suggested the close proximity and likely interaction of its single N-MBD with the A-domain (37). This was not observed when testing the co-purification of isolated domains. However, the different approaches and model proteins might be responsible for the apparent discrepancy.

Unidirectional Cu^+ Transfer from CopZ to CopA TM-MBS—We have established that, under equilibrium conditions, Ct-CopZ fully transfers Cu^+ to TM-MBS. Then, although a formal equilibrium must exist (2CopZ·Cu^+ + CopA TM-MBSs↔ 2CopZ + CopA TM-MBSs·$(Cu^+)_2$), this is highly displaced toward the CopA TM-MBSs·$(Cu^+)_2$ form making this transfer essentially unidirectional and irreversible. In agreement with this model, excess of apo-CopZ does not affect the rate of ATPase activity (turnover conditions), suggesting that apo-CopZ cannot "compete" with CopZ·Cu^+ for binding sites in CopA (34). In those experiments, although a "kinetic" effect can be argued (*i.e.* the enzyme moves into a non-interacting conformation faster than apo-CopZ binds to it), the virtually irreversible transfer of Cu^+ to TM-MBS appears as the most reasonable explanation.

Cu^+ binds to TM-MBS with very high affinity (9). This was a predictable observation in consonance with the need to prevent backward metal release from cytoplasmic sites. On the contrary, the lack of metal exchange between CopZ and CopA TM-MBS is an unexpected result with significant implication on the control of cytoplasmic Cu^+. In this direction, we might consider that the level of chaperone·Cu^+ would not control the rate of transport, and the large affinity of the ATPases for the

[3] M. González-Guerrero and J. M. Argüello, unpublished results.

chaperone·Cu^+ will not control the level of metal, as in the case of alkali metal transporters. In P_{II}-type ATPase, ATP drives conformational changes associated with gate opening exposing ion binding sites with alternative metal affinities. These affinities participate in controlling the magnitude of the transmembrane gradients and are directly linked to the energetics of the transport. This conceptual description is based on the reversible ion-protein interaction in sites at both sides of the membrane (*i.e.* affinities in the millimolar/micromolar range). Two significant concepts emerge: First, Cu^+-ATPases do not buffer the level of CopZ·Cu^+ but tend to keep it at a minimum. As a corollary, control of the number of transporters would be the mechanism buffering the cytoplasmic CopZ·Cu^+ levels. This conceptual shift explains the key role of transcriptional control in prokaryotes (46) and Cu^+-ATPases trafficking in eukaryotes (14) for Cu^+ homeostasis. Second, the transport free energy might not be simply described as a function of the transported solute gradient (*i.e.* $[Cu^+_{out}/Cu^+_{in}]$) and the membrane potential. Evaluation of this parameter will include among other factors the presence of putative (periplasmic/luminal) metal-binding proteins, the affinity for Cu^+ of outwardly facing TM-MBSs (still to be determined), etc.

Independent Loading of TM-MBSs—The occupancy of single TM-MBS upon delivery by CopZ·Cu^+ in the absence of ligands suggested the possibility to identify the sequence of TM-MBS loading and the molecular mechanism for Cu^+ release from the chaperone. Unexpectedly, measurements of CopZ-mediated Cu^+ transfer to mutated TM-MBSs suggest that both sites can be independently loaded. Moreover, the integrity of one site is not a requirement for chaperone-mediated loading of the other. The metal exchange among Cu^+ chaperones and cytosolic Cu^+ acceptor has been elegantly described by a mechanism of ligand swap involving intermediates in which ligands from both proteins participate (42, 43). A similar mechanism cannot be drawn for the transfer into TM-MBSs. However, it might be speculated that this transfer would likely occur in a protein cavity where water is absent (leading to stronger electrostatic interactions) and perhaps other groups might be available for metal removal from the chaperone in the vestibule of TM-MBS (see below).

Hypothetical Mechanism of Chaperone-mediated Cu^+ Loading of TM-MBSs—Data presented here and in previous reports (34) indicate that loading of transmembrane transport sites occurs via direct interaction of the chaperone·Cu^+ in the immediate vicinity of the TM-MBSs, that is, without participation of N-MBDs. Our results suggest an initial interaction of the chaperone·Cu^+ with the enzyme driven by complementary electrostatic interactions. Here, Cu^+ is determinant, because the apo-chaperone apparently does not interact with the ATPase. Upon release of Cu^+, the electrostatic complementarity is disrupted by conformational changes in both proteins. If the nucleotide is absent, the resulting $E1'(Cu^+)$ form cannot interact with the second chaperone·Cu^+. However, it is likely that the nucleotide-bound ATPase is the receiving form for the sequential interaction of both chaperones·Cu^+. Then, the change that drives the exit of the apo-chaperones (particularly the first one) is the modification of the chaperone surface upon Cu^+ release. We think that the shape and electrostatic of both proteins surfaces may explain the specificity and transient nature of the interaction.

How is Cu^+ released from the chaperone, and why can it indistinctly bind either TM-MBS? Among other factors, it appears likely that the interaction with the ATPase might reduce the chaperone affinity for Cu^+. Mutation of conserved residues proximal to the chaperone Cu^+-binding residues significantly increases the rate and extent of transfer to accepting chelators (47). Similarly, displacement of these key residues during the chaperone-Cu^+-ATPase interaction might facilitate the delivery to TM-MBSs. This might also be enabled by the presence of transient ligands within the chaperone-ATPase interface. These would take Cu^+ from the chaperone forming an intermediary complex and then transfer the metal to any of the two sites. This Cu^+ handling would occur at the entrance of the transmembrane path within a few angstroms of the TM-MBSs. Putative candidates for this role are residues in the cytoplasmic end of transmembrane segments forming the TM-MBSs. We have described the presence of invariant Pro (in H6) and two Asn (one in H7 and other in H8) in this location (20). Interestingly, mutation of these Asn residues alters enzyme turnover without apparently affecting Cu^+ binding (33). Although further experiments are required to test these ideas, it is probable that the reduced accessibility of TM-MBSs might require novel mechanisms of metal movement within the protein.

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