Mechanism of ATPase-mediated Cu\(^{2+}\) Export and Delivery to Periplasmic Chaperones

THE INTERACTION OF ESCHERICHIA COLI CopA AND CusF*

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Background: It is unknown how soluble chaperones acquire Cu\(^{2+}\) for delivery to metalloproteins and transporters.

Results: Cu\(^{2+}\) transfer from a Cu\(^{2+}\) efflux ATPase to a periplasmic chaperone was observed.

Conclusion: Specific transfer occurs after protein-protein recognition and interaction.

Significance: These findings explain the requirement of multiple homologous transporters and chaperones for specificity in Cu\(^{2+}\) delivery to alternative protein targets.

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Cellular copper homeostasis requires transmembrane transport and compartmental trafficking while maintaining the cell essentially free of uncomplexed Cu\(^{2+}/^{+}\). In bacteria, soluble cytoplasmic and periplasmic chaperones bind and deliver Cu\(^{2+}\) to target transporters or metalloenzymes. Transmembrane Cu\(^{2+}\)-ATPases couple the hydrolysis of ATP to the efflux of cytoplasmic Cu\(^{2+}\). Cytosolic Cu\(^{2+}\) chaperones (CopZ) interact with a structural platform in Cu\(^{2+}\)-ATPases (CopA) and deliver copper into the ion permeation path. CusF is a periplasmic Cu\(^{2+}\) chaperone that supplies Cu\(^{2+}\) to the CusCBA system for efflux to the extracellular milieu. In this report, using Escherichia coli CopA and CusF, direct Cu\(^{2+}\) transfer from the ATPase to the periplasmic chaperone was observed. This required the specific interaction of the Cu\(^{2+}\)-bound form of CopA with apo-CusF for subsequent metal transfer upon ATP hydrolysis. As expected, the reverse Cu\(^{2+}\) transfer from CusF to CopA was not observed. Mutation of CopA extracellular loops or the electropositive surface of CusF led to a decrease in Cu\(^{2+}\) transfer efficiency. On the other hand, mutation of Met and Glu residues proposed to be involved in directed Cu\(^{2+}\) transfer from transmembrane transporters. Furthermore, by explaining the movement of Cu\(^{2+}\) from the cytoplasmic pool to the extracellular milieu, these data support a mechanism by which cytoplasmic Cu\(^{2+}\) can be precisely directed to periplasmic targets via specific transporter-chaperone interactions.

Copper is an essential micronutrient required in many biological processes. It plays important catalytic roles as a prosthetic group in different enzymes (1–3). However, it also can be toxic due to its participation in Fenton chemistry and interference in [Fe-S] protein assembly (4, 5). Total intracellular Cu\(^{2+}\) levels (10–100 \(\mu\)M) are tightly controlled by transcriptional regulators, transmembrane transporters, metallochaperones, and small Cu\(^{2+}\) binding molecules (6–8). Perhaps more importantly, the high binding affinities of the cellular machinery prevent the release of Cu\(^{2+}\) into the various cellular compartments, resulting in the effective absence of free Cu\(^{2+}\) (6, 9). In consequence, Cu\(^{2+}\) movement appears mediated via ligand exchange upon protein-protein interaction. Examples of this process included metallation of Cu,Zn-SOD by the chaperone CCS, metal delivery by the bacterial chaperone CopZ to the transmembrane Cu\(^{2+}\)-ATPase for cytoplasmic efflux, and periplasmic Cu\(^{2+}\) transfer from the chaperone CusF to CusB (9–11). However, there is no evidence of how compartmental chaperones acquire Cu\(^{2+}\). These ideas suggest the existence of networks of metal distribution systems that channel the metal ions to specific compartments or target cuproproteins. Along this line, genomic analyses have shown the presence of multiple homologous cytoplasmic and periplasmic chaperones and transmembrane transporter ATPases (12–14). How are these integrated and connected?

P\(_{1B-1}\)-type Cu\(^{2+}\)-ATPases use the energy provided by ATP hydrolysis to drive the efflux of cytoplasmic Cu\(^{2+}\) across cell membranes (15, 16) and are required for maintaining cytoplasmic Cu\(^{2+}\) levels (1, 7). Recently, the metallation of membrane and periplasmic cuproproteins has been linked to their pumping of Cu\(^{2+}\) into the bacterial periplasmic space (14, 17, 18). The P-type ATPases follow the well described E1/E2 Albers-Post catalytic cycle for the translocation of substrate (16) (Fig. 1). In this cycle, the enzymes assume defined E1, E1P\(_{2}\) (Cu\(^{2+}\))\(_{2}\), E2P, and E2 conformations that can be isolated in the presence of established ligands. Structurally, Cu\(^{2+}\)-ATPases contain eight transmembrane helices (TM),\(^{3}\) cytoplasmic ATP binding and

\(^{3}\) The abbreviations used are: TM, transmembrane helix; N-MBD, N-terminal metal binding domain; MBS, metal binding site; TM-MBS, transmembrane metal binding site; DDM, n-dodecyl-\(\beta\)-D-maltopyranoside; Strep, streptavidin; PL, periplasmic loop(s).

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catalytic domains, and N-terminal regulatory metal binding domains (N-MBDs) (Fig. 2A). Conserved residues in the TM region constitute two trigonal planar Cu\(^+\) binding sites (TM-MBS) responsible for metal translocation across the permeability barrier (19). ATPases (CopA) acquire Cu\(^+\) directly from a cytoplasmic chaperone (CopZ) (10). The crystal structure of LpCopA, the Cu\(^+\)-ATPase from *Legionella pneumophila*, revealed interesting features associated with the transfer of Cu\(^+\) from the cytoplasmic chaperone (20, 21). The second TM helix of CopA is kinked, forms an electropositive platform, and exposes three Cu\(^+\)-ligating invariant residues at the cytoplasm-membrane interphase (20) (Fig. 2). This observation led to the demonstration of a detailed mechanism where the conserved platform in CopA serves as the docking site for the Cu\(^+\)-loaded CopZ. The chaperone in turn releases the Cu\(^+\) to the transiently ligating conserved Met and Glu of CopA, which form the entrance of the metal permeation path (21). However, the mechanism for Cu\(^+\) release from the ATPase to the vesicular, periplasmic, or extracellular compartments has not been established. It is logical to assume that upon ATP hydrolysis, Cu\(^+\) is outwardly released into a permeation path. A mechanism has been proposed based on the LpCopA crystal structure, in which the residues at the end of this path, the invariant Glu189 and Met100 and the less conserved Glu99 and Met214, might participate in the release of the Cu\(^+\) (20). However, considering that even in the bacterial periplasm, no free Cu\(^+\) should be available (8), we hypothesized that it is likely that in *vivo*, the transporter would directly transfer its metal to a periplasmic Cu\(^+\) chaperone.

The periplasmic Cu\(^+\) chaperone CusF is part of the CusCFBA complex responsible for the cellular Cu\(^+\) gradient (22). CusCBA forms a multiprotein complex that spans the plasma membrane, periplasm, and outer membrane. CusA is located in the plasma membrane and couples inward proton movement to metal efflux (23). CusB is a periplasmic protein that links CusA and CusC. CusC is located in the outer membrane. Together, CusCBA form a continuous channel that exports Cu\(^+\) to the extracellular space. Structurally, CusF forms a five-stranded \(\beta\)-barrel fold that binds Cu\(^+\) via conserved His\(^+\) Met, Met and Met (24). CusF acts as a chaperone to deliver Cu\(^+\) to CusB (11); however, there is no evidence to indicate how CusF is metallated *in vivo*. It is tempting to hypothesize that CusF obtains Cu\(^+\) from the corresponding Cu\(^+\)-ATPase. The *Escherichia coli* CopA (EcCopA) and CusF (EcCusF) system provides a framework to test these ideas. In this work, using *in silico* docking, Cu\(^+\) transfer determinations, co-immunoprecipitation, enzymatic analysis, and site-directed mutagenesis, we show evidence supporting the specific EcCusF-EcCopA(Cu\(^+\))\(_2\) interaction and a mechanism of metal export by Cu\(^+\)-ATPases that concludes in Cu\(^+\) transfer to periplasmic chaperones.

**EXPERIMENTAL PROCEDURES**

*Bioinformatics Analysis*—Homology modeling of EcCopA was performed using SWISS-MODEL software (25) and LpCopA (Protein Data Bank entry 3RFU) as a template. Docking of the apo-EcCusF (Protein Data Bank entry 1ZEQ) or the holo-EcCusF (Protein Data Bank entry 2VB2) structures with the periplasmic loops of the ATPase (PL1, Pro\(^+\)Gly\(^+\)225; PL2, Ser\(^+\)767–Ser\(^+\)289; PL3, Val\(^+\)850–Leu\(^+\)868; PL4, Asn\(^+\)785–Gly\(^+\)186) was analyzed with ClusPro version 2.0 using standard parameters (26, 27).

*Mechanism for Cu\(^+\) Delivery to Periplasmic Chaperones*—Cu\(^+\) was used as a template to amplify the Ec\(\Delta\)N-copA cDNA lacking the N-MBD-coding regions. This region was ligated into the pBAD-TOPO/His vector (Invitrogen), which adds an N-terminal His\(_6\) tag sequence. Ec\(\Delta\)N-copA was used as a template to introduce the mutations coding for the single substitutions M204A, M204C, E287A, and E287C and the multiple replacements D207A/N208A/M209A/M210A (M1L1), T212A/D214A/N215A/S217A (M1L2), W273A/W276A/F277A (M1L2), and W797A/T800A/T802A (M1L4). EcCusF in the pASK vector, which places a streptavidin (Strep) tag at the C terminus, was used in these studies (28). This construct was used as a template to introduce mutation coding for the multiple replacement K23A/K30A/K31A/H35A/R50A (EcCusF-5Ala). All mutations were introduced employing a QuikChange\(^{TM}\) site-directed mutagenesis kit (Agilent Technology). The sequences of primers used in this study are available upon request. DNA sequences were confirmed by automated sequencing. EcCusF carrying mutations of residues on the metal binding site (EcCusF-M471/M491) was described previously (28).

Plasmids coding for Ec\(\Delta\)N-CopA proteins were transformed into *E. coli* DC194 cells lacking endogenous EcCopA (29). Cells were grown at 37 °C in 2 × YT medium supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin. Expression of Ec\(\Delta\)N-CopA proteins was performed according to an autoinducing medium protocol (30). Plasmids carrying EcCusF, EcCusF-M471/M491, or EcCusF-5Ala cDNAs were transformed into *E. coli* BL21(DE3) strain and grown at 37 °C on LB medium supplemented with 100 μg/ml ampicillin. Expression of EcCusF proteins was induced with 200 μg/liter anhydrotetracycline hydrochloride for 6 h at 30 °C. Heterologous expression of Af\(\Delta\)N, C-CopA and AfCt-CopZ has been reported previously (10).

Purification of membrane and soluble proteins was carried out as described (10, 28, 31). Solubilized lipid/detergent micellar forms of Ec\(\Delta\)N-CopA proteins were stored in 25 mM Hapes (pH 8.0), 50 mM NaCl, 10 mM ascorbic acid, 0.01% *n*-dodecyl-\(\beta\)-d-maltopyranoside (DDM), and 0.01% asolectin (Buffer C) until use. EcCusF proteins were stored in Buffer W containing 100 mM Tris, pH 8, and 150 mM NaCl. Protein concentrations were determined by Bradford assay (32). Molar protein concentrations were estimated assuming that the proteins were pure, using \(M_\text{r} = 71,000\) for Ec\(\Delta\)N-CopA and \(M_\text{r} = 12,000\) for EcCusF. In order to eliminate any bound metal, all purified proteins were treated with metal chelators as described previously (33). Briefly, the proteins were incubated for 45 min at room temperature with 0.5 mM EDTA and 0.5 mM tetrathiomolydate in either Buffer C (ATPases) or Buffer W (CusF). Chelators were removed by buffer exchange using either 30 kDa (ATPases) or 3 kDa (CusF) cut-off Centricrons (Millipore, Billerica, MA). The final purity of all protein preparations was at least 95%, as verified by SDS-PAGE followed by Coomassie Brilliant Blue staining (data not shown).

*Cu\(^+\) Loading to Proteins*—Cu\(^+\) loading was performed by incubating each apoprotein (10 μM) in the presence of 20 μM CuSO\(_4\), 25 mM Hapes (pH 8.0), 150 mM NaCl, and 1 mM tris(2-carboxyethyl)phosphine for 10 min at room temperature with...
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gentle agitation. The unbound Cu\(^{2+}\) was removed by washing in 30 kDa (ΔN-CopA) and 3 kDa (CusF) cut-off Centricons, and proteins were used immediately. Levels of Cu\(^{2+}\) bound were verified by atomic absorbance spectroscopy (Varian). Briefly, before determinations, sample aliquots were mineralized with 35% HNO\(_3\) (trace metal grade) for 1 h at 80 °C, and digestions were concluded by making the reaction 3% H\(_2\)O\(_2\).

ATPase Assays—These were performed at 37 °C in a medium containing 50 mM Tris, pH 6.8, 3 mM MgCl\(_2\), 3 mM ATP, 0.01% asolectin, 0.01% DDM, 200 mM NaCl, 2.5 mM DTT, 20 mM cysteine, and 0.01 mg/ml purified protein. Free Cu\(^{2+}\) concentrations were varied to obtain curves reporting \(K_{iJ}\) and \(V_{\text{max}}\). ATPase activity was measured after 20 min of incubation. Released phosphate was determined according to Lanzetta et al. (34). ATPase activity measured in the absence of metal was subtracted from plotted values. Curves of ATPase activity versus free Cu\(^{2+}\) concentration were fit to \(v = \left(\frac{V_{\text{max}}[\text{Cu}^{2+}]}{[\text{Cu}^{2+}] + K_{iJ}}\right)\) using KaleidaGraph software (Synergy).

Cu\(^{2+}\) Transfer Assays—Cu\(^{2+}\) transfer from various EcΔN-CopA constructs to different Strep-tagged EcCusF constructs was performed following protocols similar to those developed to characterize metal transfer from cytoplasmic chaperones to ATPases (10). Briefly, 10 μM holo-His-tagged EcΔN-CopA (or ΔfΔN,C-CopA) was incubated in the presence of the indicated molar excess of Strep-tagged EcCusF (or AfCt-CopZ) and subsequently bound to a Strep-tactin column in buffer containing 25 mM Hepes (pH 8.0), 150 mM NaCl, 0.01% asolectin, 0.01% DDM, 100 μM bicinchoninic acid, and 1 mM tris(2-carboxyethyl)phosphine. Cu\(^{2+}\) transfer was initiated by the addition of 100 μM ATP-MgCl\(_2\) and incubated for 2 min at room temperature. In control experiments, ATP was substituted by 100 μM ADP. Proteins were separated by washing the Strep-tactin column with 25 mM Hepes (pH 8.0), 150 mM NaCl, 0.01% DDM, 0.01% asolectin, 1 mM tris(2-carboxyethyl)phosphine, followed by elution in the same buffer supplemented with 2.5 mM des-thiobioc. Cu\(^{2+}\) content in each fraction was measured by furnace atomic absorbance spectroscopy, as indicated above. Control experiments were performed using apoproteins, and observed Cu\(^{2+}\) levels were subtracted. Controls were performed where Cu\(^{2+}\)-loaded EcCusF/AfCt-CopZ Strep and EcΔN-CopA were subjected to the same procedures individually (i.e. lacking a partner Cu\(^{2+}\)-exchange protein). Proteins eluting in each fraction were determined by SDS-PAGE followed by Western blot using anti-His\(_6\) (Genescript), anti-EcCusF (Lampire Biological Laboratory), or Strep-tactin (IBA) rabbit antibodies.

Immunoprecipitation—20 μM concentrations of micellar apo- and holo-forms of EcΔN-CopA were incubated for 2 h with 20 μM EcCusF in buffer containing 25 mM Tris, pH 6.8, and 25 mM DTT. 100 μM ADP-MgCl\(_2\), 3 mM K\(_2\)HPO\(_4\)-MgCl\(_2\), or 100 μM Na\(_2\)VO\(_4\)-MgCl\(_2\) were included as indicated in Fig. 5. Anti-EcCusF antibody was added, and the samples were incubated for 2 h at 4 °C. Protein-antibody complexes were incubated overnight with pre-equilibrated Protein A/G-agarose resin (Roche Applied Science). Nonspecific interactions were removed by washing with 25 mM Tris, pH 7.2, and 150 mM NaCl. The EcΔN-CopA-EcCusF complexes were separated from the antibody-agarose beads in freshly prepared buffer containing 50 mM Tris, pH 6.8, 10% glycerol, 1 mM NaCl. IgS bound to the Protein A/G-agarose beads was removed by elution with buffer containing 0.2 M glycine HCl, pH 2.5 (data not shown). The protein content in these washes was analyzed by SDS-PAGE followed by Western blot using anti-His\(_6\) and anti-EcCusF antibodies as described above.

RESULTS

Molecular Docking of CusF with CopA Extracellular Loops—We began our studies by analyzing in silico the probable interacting regions of EcCopA and the periplasmic Cu\(^{2+}\) chaperone EcCusF. The extracellular loops of EcCopA, based on the structure of LpCopA (Protein Data Bank entry 3RFU), and the crystal structure of apo-EcCusF (Protein Data Bank entry 1ZEQ) were used for docking analysis with ClusPro software. ClusPro implements a multistage protocol: rigid body docking, energy-based filtering, ranking the retained structures based on clustering properties, and finally, the refinement of a limited number of structures by energy minimization. The model presented in Fig. 2 was selected after considering the lower energies and larger cluster size among other similarly models. The model reveals the proximity of residues proposed to be involved in Cu\(^{2+}\) release from the ATPase (Met\(^{204}\) and Gly\(^{287}\)) (20) with the EcCusF metal binding site (MBS) (His\(^{36}\), Met\(^{67}\), and Met\(^{98}\)). This is interesting, taking into account that the structure of LpCopA was obtained for the enzyme in the E2P conformation (20). This conformation might interact less strongly with EcCusF than the E1 forms (E1-ATP-(Cu\(^{2+}\))\(_2\) or E1P-(Cu\(^{2+}\))\(_2\) (Fig. 1). Analysis attempting the docking of EcCopA with the holo-EcCusF (Protein Data Bank entry 2VB2) structure did not produce reasonable dockings because none of the models returned by ClusPro placed the Cu\(^{2+}\) exit site of the ATPase in proximity to the EcCusF MBS. In these models, residues 53TPQTKMSEIK\(^{60}\) or 74QQGNLS\(^{80}\) of EcCusF (opposite to the MBS) interacted with periplasmic loop 3 and, to a lesser extent, with loop 4 of the ATPase. Energy values were higher and cluster size was smaller for these structures compared with those for apo-EcCusF/CopA interaction models (data not shown). The lack of interactions of EcCusF-(Cu\(^{2+}\)) with the ATPase supports a functional mechanism where, upon receiving Cu\(^{2+}\), the chaperone detaches from the ATPase.

CopA Delivers Cu\(^{2+}\) to CusF—The functional interaction of EcCopA and EcCusF was explored by testing Cu\(^{2+}\) transfer among the isolated proteins. We hypothesized that a stable...
The capability of EcCusF (Fig. 3) to characterize metal transfer from the cytoplasmic Cu

A. fulgidus CopA) was used to prevent any interference by metal binding to truncated version of EcCopA lacking the N-MBS (EcCopA/H9004).

The ATPase catalytic conformations and binding stoichiometry of E. coli CopA wild type and mutants

| E. coli CopA | $V_{max}$ | $K_{m}$ | Cu$^+/protein molar ratio$^a |
|-------------|----------|-------|---------------------|
| Wild type   | 1.64 ± 0.27$^b$ | 1.48 ± 0.60 | 2.1 ± 0.1 |
| ΔN.C-CopA   | 1.45 ± 0.29 | 1.32 ± 0.40 | 2.1 ± 0.1 |
| M1L1        | 1.18 ± 0.18 | 0.73 ± 0.44 | 2.1 ± 0.1 |
| M2L1        | 1.16 ± 0.23 | 1.39 ± 0.75 | 2.2 ± 0.3 |
| M1L2        | 0.98 ± 0.11 | 0.63 ± 0.27 | 2.1 ± 0.2 |
| M2L2        | 1.11 ± 0.10 | 0.41 ± 0.21 | 2.1 ± 0.1 |
| M1L4        | 1.54 ± 0.19 | 1.25 ± 0.37 | 2.2 ± 0.1 |
| M204A       | 0.72 ± 0.13$^c$ | 0.50 ± 0.27$^c$ | 2.2 ± 0.1 |
| M204C       | 1.46 ± 0.30 | 1.72 ± 0.10 | 2.0 ± 0.1 |
| E287A       | 0.74 ± 0.13$^c$ | 0.52 ± 0.11$^c$ | 2.2 ± 0.3 |
| E287C       | 1.32 ± 0.06 | 1.28 ± 0.16 | 2.1 ± 0.1 |

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

$^b$ Values are the best fit parameters of activity versus [Cu$^+$] (mean ± S.E.) ($n = 3$).

$^c$ p ≤ 0.05. Significant differences from the kinetic parameters observed from EcΔN-CopA were determined by Student’s t test.

results suggest that the CusF-CopA affinity might be relatively weak, and for complete transfer to occur, excess EcCusF should be present in the system. Supporting this idea, Fig. 3C shows the metal transfer when a 10-fold molar excess of EcCusF was present. Notice that because the experiments were performed with large amounts of protein, the EcCusF peak broadens, and there was a significant amount of Cu$^+$ eluting in the third and fourth elution fractions. The levels of Cu$^+$ eluting in the initial washing of the columns were minimal (Fig. 3C). Fig. 3E shows more clearly the relationship between Cu$^+$ transfer and the EcΔN-CopA/EcCusF ratio and the largely complete transfer when EcCusF is in excess.

Specific Cu$^+$ transfer from the ATPase to EcCusF is expected to be dependent on ATP hydrolysis and direct protein-protein interactions. In the presence of ATP, all Cu$^+$ is released from the ATPase. The dependence of metal transfer on ATP hydrolysis was verified by replacing the trinucleotide with ADP, which resulted in no observable Cu$^+$ transfer (Fig. 4, A and C). An intact EcCusF metal binding site is important for metal transfer because an EcCusF mutant incapable of binding Cu$^+$ (EcCusF-M47I/M49I) (EcCusF, 0.96 ± 0.06; EcCusF-M47I/M49I, 0.08 ± 0.02 Cu$^+$/protein molar ratio; $n = 3$) (22) but still folding similarly to wild type protein was unable to receive Cu$^+$ from EcΔN-CopA (Fig. 4, A and C). To determine whether specific protein-protein interactions are required for metal transfer, EcCusF was substituted by a cytosolic chaperone from Archaeoglobus fulgidus, AfCt-CopZ. Cu$^+$ transfer among these two proteins was not detected (Fig. 4, B and C). Correspondingly, no Cu$^+$ transfer to EcCusF was detected if EcΔN-CopA was replaced by A. fulgidus AfΔN.C-CopA (Fig. 4, B and C).

Effects of the ATPase Catalytic Conformations on the CopA-CusF Interaction—The experiments described above suggest that EcCusF interacts with the ATPase primarily when the enzyme assumes an E1 conformation (ATP-E1([Cu$^+$])$_2$ or E1P([Cu$^+$])$_2$) (Fig. 1). Following this logic, it is likely that CopA in a non-turnover E1 form could stably bind CusF. Testing this hypothesis, EcΔN-CopA and EcCusF were allowed to interact under different conditions and then immunoprecipitated using an antibody against EcCusF, and the Western blots were developed with an anti-His$_6$ antibody to detect EcΔN-CopA.
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**Fig. 5**, lane 3, shows the co-precipitation of Ec\(\Delta N\)-CopA\((\text{Cu}^{+})_2\) (i.e. E1-metal) with apo-EcCusF in the absence of any other ligand. Moreover, inclusion of ADP, placing the ATPase in E1-nucleotide(metal) form, led to an increase in Ec\(\Delta N\)-CopA precipitation (Fig. 5, lane 4). Interestingly, the interaction was present even in the absence of Cu\(^{+}\) because Ec\(\Delta N\)-CopA is in an E1β→E2 equilibrium (Fig. 5, lane 5). Alternatively, the interaction was largely prevented if Ec\(\Delta N\)-CopA was driven into an E2 form either by inclusion of \(\text{P}_i\)-Mg\(^{2+}\) or Na\(_3\)VO\(_4\)-Mg\(^{2+}\) in the absence of Cu\(^{+}\) (Fig. 5, lanes 6 and 7). Finally, as expected, the interaction between holo-EcCusF and apo-Ec\(\Delta N\)-CopA was minimal (Fig. 5, lane 8). These results provide further evidence for specific protein-protein interactions mediating the Cu\(^{+}\) transfer to the apo-chaperone upon transport of the metal through its permeation path (Fig. 1).

**Structural Determinants of the CopA-CusF Interaction**—Electrostatic interactions appear particularly relevant for the interaction of the cytoplasmic Cu\(^{+}\) chaperones with Cu\(^{+}\)-ATPases and subsequent metal delivery for transmembrane transport (21). Additionally, positively charged residues in CusF appear to be important for interactions with the periplasmic adaptor protein CusB (11). Electrostatic interactions are probably important for protein-protein interactions between EcCopA and EcCusF on the exit side of the metal permeation path. In support of this suggestion, EcCusF presents a number of electropositive side chains on its surface, which appear close to the periplasmic loops of EcCopA in the docking models.
Mechanism for Cu⁺ Delivery to Periplasmic Chaperones

Cu⁺ (16). Fig. 7D shows that replacements in extracellular loops in EcΔN,C-CopA have distinct effects in Cu⁺ transfer to EcCusF. Here, the results of the transfer experiments (similar to those shown in Fig. 3B) are presented as the percentage of Cu⁺ eluted with the ATPase or the chaperone. Replacement of polar residues in the N-terminal region of PL1 (M1L1) abolished the transfer of Cu⁺ to EcCusF upon activation by ATP; however, mutation of residues at the C-terminal portion of this loop (M2L1) apparently did not affect the interaction. On the other hand, PL2 and PL4 mutant proteins yielded less marked but noticeable decreases in Cu⁺ transferred to EcCusF (Fig. 7D). These results support the idea that the protein-protein interaction is mediated by specific structural components in the ATPase.

Role of the Putative Transient Exit Site in Cu⁺ Release and Transfer to CusF—Gourdon et al. (20) first noticed invariant residues located close to the exit of the permeation path of Cu⁺-ATPases (Fig. 2A). They hypothesized that these might be instrumental in metal release. Sequence comparison and molecular modeling of EcCopA versus LpCopA showed that EcCopA Met204 and Glu287 correspond to the invariant residues postulated as a putative “exit” site. To test the importance of these residues in metal delivery to the periplasmic chaperone, Met204 and Glu287 were replaced by Ala or Cys in the EcΔN-CopA background. The non-conservative Ala replacement led to a marked (50%) reduction in activity that was not evident in the Cys mutants (Fig. 8, A and B, and Table 1). This is an interesting observation because metal release (and the associated transition to E2) is rate-limiting under turnover conditions (16, 31). As observed in proteins carrying alterations in extracellular loops, mutation of Met204 and Glu287 decreased the Cu⁺ K₅₀ for activation without altering the binding stoichiometry (Fig. 8, A and B, and Table 1). Additional evidence for the participation of these residues in metal release was provided by Cu⁺ transfer experiments. In these cases, Ala substitutions rendered a partial decrease in the amount of Cu⁺ transferred to CusF (Fig. 8C), whereas conservative Cys mutations behaved similarly to EcΔN-CopA (Fig. 8C). These results suggest that these residues may transiently interact with the metal, facilitating its release to the acceptor protein.

DISCUSSION

It is now well accepted that Cu⁺ homeostasis requires its distribution and a detailed control of delivery by metal chaperones to correct target cuproenzymes or efflux transporters in the various cellular compartments (8, 36). However, it is remarkable that there is no description of how soluble chaperones acquire Cu⁺. In addition, the potential toxicity of Cu⁺ requires that Cu⁺ distribution be mediated by protein-protein interaction with Cu⁺ transfer through a ligand exchange mechanism. Again, there is limited experimental evidence of this conceptual description. Metallation of Cu,Zn-SOD by the chaperone CCS provided an early example of this process (9). Since then, our groups have shown metal delivery by the bacterial chaperone CopZ to the transmembrane Cu⁺-ATPase for cytoplasmic efflux (10, 21) and Cu⁺ transfer in the periplasm from the chaperone CusF to CusB (11). Linking these last two phenomena, here we present experimental evidence of specific

![Figure 6.](image-url)
Cu⁺ transfer from an efflux ATPase to a soluble Cu⁺ chaperone. Based on this finding, it is now possible to fully understand the movement of Cu⁺ from the cytoplasmic pool to the extracellular milieu and hypothesize Cu⁺ distribution networks supported by various homologous transporters and chaperones.

**Cu⁺ Transfer Is Mediated by Protein-Protein Interactions**—Cu⁺ exchange between cytoplasmic metallochaperones and the regulatory N-MBDs of Cu⁺-ATPases provided early support for a mechanism of metal transfer involving molecular recognition and ligand exchange (37–40). In these cases, equilibrium between the proteins ($K_{eq}$) is established in accordance with the regulatory roles of the N-MBDs (41, 42). Biochemical characterization of chaperone-mediated Cu⁺ delivery to both TM-MBSs present in Cu⁺-ATPases showed again a process where protein-protein interaction confers specificity and subsequent ligand exchange enables metal transfer (21). However, in this case, the transfer was...
stoichiometric (i.e. no equilibrium is detectable), providing a molecular blockage for backward metal flux (10, 35). Studies of the CusF system have demonstrated specific metal transfer between the CusF and CusB proteins, which occurs bidirectionally between these two proteins in vitro. Copper efflux from the assembled CusCBA transport system is expected to result in unidirectional Cu\(^{2+}\) efflux in vivo. The data presented here show that the soluble Cu\(^{2+}\) chaperone CusF obtains metal from the transport Cu\(^{2+}\)-ATPase in an analogous manner. Transfer is achieved through a specific protein-protein interaction because replacement of either component by an alternative protein prevents the transfer. Modeling of the complex suggests the proximity of metal binding sites in both proteins, which would enable ligand exchange. However, distinct from previous examples, transfer requires an excess of the accepting chaperone in the medium. This implies that in vivo the apo-chaperone species must predominate. Microarray studies accepting chaperone in the medium. This implies that the transfer appears dependent on ATP hydrolysis rather than the presence of nucleotide. In addition, the various conformations that the enzyme adopts during catalysis interact differentially with the chaperone, and the pump does not interact with holochaperone. Cu\(^{2+}\)-ATPases follow the Post-Albers catalytic cycle (Fig. 1). In the E1 conformation, cytoplasmic facing TM-MBS have high affinity for Cu\(^{2+}\). Upon binding of two Cu\(^{2+}\), the ATPase is phosphorylated and transitions to the E1-P state, where Cu\(^{2+}\) ions are probably in an occluded state. Subsequently, the enzyme moves into the E2 form, where the outwardly facing TM-MBS have lower affinity for Cu\(^{2+}\), leading to Cu\(^{2+}\) release. Consequently, it appears logical that apo-CusF would selectively interact with E1-Cu\(^{2+}\) forms of the enzyme in order to trap the released metal. This is supported by our co-immunoprecipitation data, in which the E1-Cu\(^{2+}\) has higher affinity for the periplasmic chaperone. During each catalytic cycle, two Cu\(^{2+}\) ions are transported. In alkaline metal-transporting ATPases, the sequential release of Na\(^{+}\) or Ca\(^{2+}\) as the enzyme transitions from E1P to E2P has been shown (44). If a similar sequential release is assumed in Cu\(^{2+}\)-ATPases, does CusF interact with an enzyme that is close to either the E1P or E2P states? Further studies are required to answer this. The presence of the chaperone does not seem to affect the transport stoichiometry because both Cu\(^{2+}\) leave the ATPase to load two CusF equivalents, provided that the chaperone is in excess. To fulfill this mechanistic requirement, the ATPase has poor affinity for the holo-CusF that leaves the enzyme surface, as shown by in silico docking and co-immunoprecipitation experiments. Thus, another apo-CusF can dock to receive the second Cu\(^{2+}\). Finally, following the cycle, the E2 forms of the ATPases have a reduced affinity for CusF, as supported by the minimal interaction in co-immunoprecipitation experiments.

**Structural Determinants of the ATPase-Chaperone Interaction**—

Our results show the physical interaction of the chaperone and ATPase proteins. It therefore appeared relevant to investigate the structural determinants of this phenomenon because they would influence the selectivity of metal transfer from a given ATPase to the correct chaperone. Consider, for instance, *Pseudomonas aeruginosa*, which has two Cu\(^{2+}\)-ATPases, one participating in Cu\(^{2+}\) efflux into the extracellular milieu and the other involved in cytochrome c oxidase assembly (17). It is clear that the fate of transported Cu\(^{2+}\) will be determined by the chaperone accepting the metal. Even more complex is *Sinorhizobium meliloti*. This organism has five Cu\(^{2+}\)-ATPases with different functions (45), two putative CusF proteins, and two SenC-like proteins chaperoning Cu\(^{2+}\) for metallation of cytochrome c oxidase. An understanding of the structural relationship among chaperones and ATPases appears critical to explain the Cu\(^{2+}\) distribution networks. Cu\(^{2+}\)-ATPases have four periplasmic loops of different lengths containing residues of diverse polarity; therefore, these loops can present a particular structure suitable for recognition and stabilization of interactions. CusF has a partially electropositive surface, which is largely conserved among CusF homologs. Some of these electropositive residues are involved in interactions with CusB (11). We observed that replacement of polar amino acids in these surfaces leads to several degrees of alteration in Cu\(^{2+}\) transfer. This appears as the plausible result of modifying one element of several contributing to stabilizing the interaction. Nevertheless, correlating in silico docking analysis and the transfer experiments, it seems clear that the first half of PL1 is critical, whereas the first half of PL2 does not contribute to the interaction. In the docked model, Lys and His residues in the chaperone surface are proximal to PL1 of the ATPase.

**Metal-ligating Residues Might Participate in Cu\(^{2+}\) Release**—

Experiments characterizing the human Cu\(^{2+}\)-ATPase ATP7A showed that its first luminal loop has a unique sequence rich in His and Met (46). Analysis of a chimeric construct of this loop with a scaffold protein showed that it binds Cu\(^{2+}\), and deletions of the His and/or Met residues inhibit dephosphorylation of the ATPase, required for the transition to E2 state and Cu\(^{2+}\) release. Interestingly, the Cu\(^{2+}\)-loaded loop is able to transfer either Cu\(^{2+}\) or Cu\(^{2+}\) to a luminal enzyme, peptidylglycine monooxygenase, suggesting a direct interaction between both proteins (46). Although these Met/His residues are absent in the periplasmic loops of bacterial Cu\(^{2+}\)-ATPases, Gourdon et al. (20) identified invariant Met/Glu residues present in the exit end of the ion permeation path of these proteins, although no stable binding of Cu\(^{2+}\) to a site formed by these residues has been observed (19). Exploring a possible role in Cu\(^{2+}\) transport and transfer, mutations of the conserved Met\(^204\) and Glu\(^{297}\) were characterized. Activation of CopA by free Cu\(^{2+}\) was significantly affected by non-conservative substitutions on the exit sites. Ala-substituted enzymes exhibited half of the control activity, and the apparent affinity of cytosolic sites increased. The effects were attenuated when Cys residues were introduced at these positions. These observations suggest that metal release from the TM-MBSs depends on the structural integrity at the exit of the permeation path independent of metal transfer to a chaperone. A mechanism where these residues accelerate Cu\(^{2+}\) movement in a water-inaccessible ion well remains to be tested. For instance, a first intermediate formed with Glu\(^{297}\) might promote outward Cu\(^{2+}\) movement. This would be the equivalent of Glu\(^{297}\) in SERCA1a, which is required for Ca\(^{2+}\) release and the proper function of the cytosolic binding sites (20, 47). Inde-
dependent of a role “pulling” the Cu⁺ along the permeation path, these residues are likely to play a role in metal delivery to the chaperone because M204A and E287A showed a reduced Cu⁺ transfer to CusF. Similarly to previous observations for chaperone-mediated Cu⁺ delivery to the ATPase, the metal transfer from the exit sites is possible in M204C- and E287C-substituted enzymes. In consequence, a mechanism involving ligand exchange-mediated Cu⁺ delivery is likely to occur to allow metal release.

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