The Architecture of CopA from *Archeaoglobus fulgidus* Studied by Cryo-Electron Microscopy and Computational Docking

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SUMMARY

CopA uses ATP to pump Cu⁺ across cell membranes. X-ray crystallography has defined atomic structures of several related P-type ATPases. We have determined a structure of CopA at 10 Å resolution by cryo-electron microscopy of a new crystal form and used computational molecular docking to study the interactions between the N-terminal metal-binding domain (NMBD) and other elements of the molecule. We found that the shorter-chain lipids used to produce these crystals are associated with movements of the cytoplasmic domains, with a novel dimer interface and with disordering of the NMBD, thus offering evidence for the transience of its interaction with the other cytoplasmic domains.Docking identified a binding site that matched the location of the NMBD in our previous structure by cryo-electron microscopy, allowing a more detailed view of its binding configuration and further support for its role in autoinhibition.

INTRODUCTION

Copper is essential to cells as a cofactor for a wide variety of enzymes such as superoxide dismutase and cytochrome oxidase. If not carefully controlled, however, Cu can be toxic, due to its redox potential and its ability to produce free radicals (Rae et al., 1999). As a result, a system of pumps, transporters, and metallochaperones has evolved to control the delivery and distribution of Cu. In general, intracellular Cu scavengers, such as metallothioneins, ensure that there is a vanishingly low concentration of free Cu in the cytoplasm (Rae et al., 1999), and metallochaperones, such as Atox1, CCS, and Sco1/2, are used to carry the Cu through the cytoplasm and deliver it to specific targets (Lutsenko et al., 2007). In mammals, Ctr1 is a secondary transporter on the cell surface that facilitates entry into the cell (Lee et al., 2001), and two transmembrane ATPases, ATP7A and ATP7B, pump Cu across the endoplasmic reticulum and plasma membrane, respectively (Lutsenko et al., 2007). Mutations in ATP7A give rise to Menkes disease, resulting from insufficient delivery of Cu in brain and other tissues, whereas mutations to ATP7B are responsible for Wilson’s disease, where Cu overload is responsible for liver and brain dysfunction (Barry et al., 2010; Veldhuis et al., 2009a).

ATP7A and ATP7B are homologous to CopA from bacteria and belong to the family of P-type ATPases (Lutsenko and Kaplan, 1995). This family comprises ATP-dependent, transmembrane ion pumps that share a common reaction mechanism, membrane topology, domain organization, as well as conserved sequence motifs implicated in ATP hydrolysis and in formation of their hallmark phosphoenzyme intermediate (Kühlbrandt, 2004; Møller et al., 1996). Phylogenetic analysis of P-type ATPases delineates five subgroups, denoted P1–P5 (Axelsen and Palmgren, 1998). CopA, ATP7A, and ATP7B are in the P1B subfamily together with transporters of a diverse array of transition and heavy metal ions such as Cu⁺, Cu²⁺, Zn²⁺, Cd²⁺, Co²⁺, and Pb²⁺.

P1B ATPases are the most thoroughly studied and have become paradigms for the family. They include Ca²⁺-ATPase and Na⁺/K⁺-ATPase, which have ten transmembrane helices (M1–10) and two main cytoplasmic loops inserted between M2/M3 and M4/M5. The larger cytoplasmic loop (between M4/M5) folds into two separate domains, thus composing the nucleotide-binding (N) domain and phosphorylation (P) domain, whereas the smaller loop (between M2/M3) composes the so-called actuator (A) domain. Each domain is characterized by sequence motifs related to their individual roles in the reaction cycle, namely DKTGTLT at the phosphorylation site of the P domain, isolated residues surrounding the ATP binding site in the N domain, and the conserved TGE in the A domain (Olesen et al., 2007). The well-characterized reaction cycle alternates between E1 and E2 states, in which transmembrane ion binding sites are oriented toward the intracellular or extracellular milieu, respectively. Switching between these two states is controlled by phosphorylation of the catalytic aspartate in the conserved DKTGTLT at the phosphorylation site of the P domain, isolated residues surrounding the ATP binding site in the N domain, and the conserved TGE in the A domain (Olesen et al., 2007).
P_{ib} ATPases such as CopA, although functionally and structurally related to P_{1} ATPases, are distinguished by having only eight transmembrane helices and by bearing one or more N-terminal metal-binding domains (NMBDs). These NMBDs are homologous to soluble metallochaperones that carry copper through the cytoplasm (e.g., Atox1). Both NMBDs and metallochaperones bind Cu^{+} with high affinity via CxxC sequence motifs. Although it has been postulated that NMBDs mediate transfer of Cu^{+} from the metallochaperones to transport sites, there is increasing evidence that these domains are instead involved in autoregulation and, in the case of ATP7A, in targeting the molecule to the basolateral membrane (see reviews by Argüello et al., 2007; Lutsenko et al., 2007). The structural basis for this autoregulation remains to be determined. X-ray structures have been solved for several intact P_{ib} ATPases, including Ca^{2+}-ATPase from sarcoplasmic reticulum (SERCA1) (Toyoshima et al., 2000), Na^{+}/K^{+}-ATPase (Morth et al., 2007), and H^{+}-ATPase (Pedersen et al., 2007). These structures have demonstrated the nature of the E1 and E2 states as well as the role of conformational changes in coordinating the multiple steps of the enzymatic cycle (Olesen et al., 2007). X-ray structures of P_{ib}-type ATPases have so far only involved isolated cytoplasmic domains of CopA from Archaeoglobus fulgidus: specifically, a construct containing the N and P domains (Szazinsky et al., 2006b; Tsuda and Toyoshima, 2009) and another construct of the A domain alone (Szazinsky et al., 2006a). NMR has revealed the binding of ATP to the isolated N domain (Banci et al., 2010) and has characterized the structural homology between various NMBDs and the soluble metallochaperones (e.g., Banci et al., 2006). Our previous reconstruction of CopA using cryo-electron microscopy (cryo-EM) is the only structure of an intact P_{ib}-type ATPase. Although the resolution was limited (~17 Å), this reconstruction served as a template for building a CopA homology model that defined the location of the NMBD relative to the other cytoplasmic domains and suggested that the NMBD could regulate CopA activity by holding the enzyme in an inactive state in the absence of copper and ATP (Wu et al., 2008). A different conclusion was reached by a more recent study employing chemical crosslinking, which suggests that the NMBD binds to the opposite side of the molecule and represents a static structural element of the cytoplasmic domain (Lübben et al., 2009).

To reevaluate the location and role of the NMBD, we have determined a higher-resolution structure using cryo-EM and helical reconstruction of tubular crystals of CopA from A. fulgidus. This 10 Å resolution structure reveals well-defined cytoplasmic domains that are readily fit with the A, N, and P domains from X-ray crystallography. Although the transmembrane domain is less distinct, the new map leads us to propose a novel location for the extra two N-terminal helices that characterize the P_{ib} subfamily. Surprisingly, the NMBD is disordered in these crystals, presumably due to dramatic changes in the molecular packing of CopA, thus supporting the idea that the NMBD is not a static structural element of the cytoplasmic domain. Based on an all-atom homology model for the cytoplasmic domains of CopA, we used computational methods to dock the NMBD, thus identifying the most favorable site of interaction. The results are consistent with our previous structure from cryo-EM and with the proposed autoinhibitory role for NMBD, which would act by retaining CopA in the E2 conformation.

### RESULTS

#### New Crystal Form for CopA

One of our primary goals was to obtain a higher-resolution structure of CopA in order to understand better the architecture of the various domains that compose the molecule. As in earlier work, we used Escherichia coli to express ΔC-CopA from A. fulgidus that carried the functionally important N-terminal MBD, which is common to all P_{ib} ATPases, but not the C-terminal MBD, which is a unique feature of this particular CopA homolog. Expression levels were quite high, producing a final yield of 6–9 mg of detergent-purified protein from a 6 l expression with purity >98% (see Figure S1 available online).

By altering the lipid species and the temperature used for crystallization, we obtained wider tubular crystals with stronger diffraction compared to our previous work. Previously, tubular crystals of ΔC-CopA (Wu et al., 2008) were produced at 50 °C with a reconstituted membrane of DOPC (1,2-dioleoyl-sn-glycery-3-phosphocholine) and in a solution containing the Cu^{+} chelator BCDS (bathocuproinedisulfonic acid) at pH 6.1. These tubes were 35 nm in diameter and the corresponding structure resembled that of SERCA in the E2 enzymatic state, consistent with the absence of Cu^{+} in the crystallization media. For the current work, the lipid was changed to a mixture of DMPC/DOPE (1,2-dimyristoyl-sn-glycero-3-phosphocholine/1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) (4:1 weight ratio), which produced substantially wider (60 nm diameter) tubular crystals at 30 °C under otherwise identical conditions (Figure 1). Layer line data from these wider crystals were substantially stronger and were consistent with the presence of several related helical symmetries, characterized by systematic differences in diameter and by the Bessel orders of principal layer lines: (n_{1},n_{0},n_{1}) equal to either (12,−8), (14,−8), or (14,−10). Among tubes with (12,−8) symmetry, the variability of unit cell dimensions for the underlying 2D lattice was small (SD <0.5 Å and <0.4 Å for the included angle) (Table 1), making the Fourier-Bessel approach to helical reconstruction viable (Diaz et al., 2010). Based on two-fold related phase residuals, the final map from an average of 12 tubes was judged to have 10 Å resolution, compared to 17 Å from a similar number of tubes in DOPC (Figure 2C). The visibility of several individual x helices in the map (discussed below) is consistent with this estimate of resolution.

#### Packing of ΔC-CopA in Tubular Crystals

The reconstruction shows that the orientation of ΔC-CopA in DMPC/DOPE tubes is inverted relative to DOPC tubes (Figure 2; Figure S2). Similar to helical crystals of Ca^{2+}-ATPase (Xu et al., 2002) and nicotinic acetylcholine receptor (Toyoshima and Unwin, 1988), ΔC-CopA molecules in DOPE tubes were oriented with their cytoplasmic domains facing the outside of the highly curved bilayer. In these cases, the positive curvature is presumably induced by the larger linear dimension required to accommodate the much larger cytoplasmic domains relative to the transmembrane and luminal domains (Young et al., 1997).

Surprisingly, the large cytoplasmic domains of ΔC-CopA face the inside of tubes produced from DMPC/DOPE. This inverted
orientation was also observed for tubular crystals of Na+/K+-ATPase grown in native membranes from the duck supraorbital salt gland, although in this case the \( \beta \) subunit added substantial mass to the outside of these tubes (Rice et al., 2001).

The packing of \( \Delta C \)-CopA molecules is also substantially different in DMPC/DOPE tubes. In both crystal forms, CopA forms dimers that are stabilized by reciprocal interactions between N and A domains. In DOPC tubes, the dimers pack into “dimer ribbons” reminiscent of Ca\(^{2+}\)-ATPase crystals (Taylor et al., 1986), in which contacts between A and N domains form a continuous ribbon of molecules related by two-fold symmetry, with another ribbon running in the opposite direction along the \( b \) axis of the unit cell (Figure 2B). In contrast, DMPC/DOPE tubes lack dimer ribbons; instead, the lattice is characterized by a more isotropic set of contacts between neighboring dimers. As will be discussed in more detail below, the geometry of the dimer itself is also quite different, with a 50°/C14 angle between the molecules. As a result of these differences, the density of cytoplasmic domains in DMPC/DOPE tubes is 33% higher than in DOPC tubes, based on the area of the unit cell at radii corresponding to the middle of these domains (Table 1).

In contrast to the cytoplasmic domains, the transmembrane domains in DMPC/DOPE tubes are less densely packed, with a density at the center of the membrane that is <60% relative to DOPC tubes (Table 1). Another way to look at this difference...
is to compare membrane and cytoplasmic domains within each crystal: the unit cell area within the membrane of DOPC tubes is considerably smaller (64%) than in the region of the cytoplasmic domains, as one would expect given the smaller size of the molecule within the membrane. In contrast, the inverted nature of the DMPC/DOPE tubes provides a much larger area to the membrane domains relative to cytoplasmic domains (150%).

This excess membrane area in DMPC/DOPE tubes may explain the diffuse density seen on the outer surface of the reconstruction (Figure 3). When images of individual tubes are projected down their cylindrical axes, several discrete peaks are observed in the resulting 1D profile (Figure 3C). At the innermost radii, these peaks correspond to cytoplasmic domains, followed by two peaks from the phosphate head groups of the lipid bilayer. Unlike previous tubes of CopA and of Ca2+-ATPase, there is an additional density peak on the outer surface of the membrane in DMPC/DOPE tubes (arrow in Figure 3C). Helical reconstruction of these tubes produces a considerable amount of low, diffuse density in this region. More organized features with somewhat higher density are visible outside the tubes, but these features lie directly along symmetry axes, where noise builds up during the reconstruction process (e.g., compare sections shown in Figures 3A and 3B). From these observations, we speculate that there is a population of disordered CopA molecules with their cytoplasmic domains facing the outside of the DMPC/DOPE tubes. Indeed, a symmetric distribution of molecules across the membrane is expected from reconstitution, and the inverted geometry of the CopA crystals within DMPC/DOPE tubes produces excess membrane area that could readily accommodate extra molecules facing the outside of the tubes. These extra molecules would be unable to participate in regular crystal contacts given the long distance between their cytoplasmic domains. Furthermore, the presence of disordered molecules would explain why, in the helical reconstruction, the protein domains within the membrane have much lower contrast relative to the cytoplasmic domains on the inside of the tube.

Functional Analysis of CopA
Because of the dramatic effect of bilayer composition on the morphology and molecular packing of the tubular crystals, we used ATPase assays to assess whether lipid had an effect on ΔC-CopA function. In particular, we characterized the dependence of ATPase activity on Cu⁺ or Ag⁺ concentrations and on temperature, reasoning that bilayer composition might affect either the mobility or the stability of the enzyme. The results (Figure 4) indicate that ΔC-CopA has an apparent K_d of 0.44 μM for Cu⁺ and 0.26 μM for Ag⁺ in DMPC/DOPE and corresponding V_max of 1.97 and 1.76 μmol/mg/min. For comparison, affinities in DOPC were 0.23 and 0.22 μM for Cu⁺ and Ag⁺ with V_max of 0.94 and 1.48 μmol/mg/min, respectively. These values are similar to those previously measured by Rice et al. (2006) from detergent-solubilized ΔC-CopA: K_d of 0.11 and 0.2 μM for Cu⁺ and Ag⁺, respectively, with V_max of 1.8 and 2.4 μmol/mg/min. In all cases, ATPase activity was partially inhibited at high concentrations of either Cu⁺ or Ag⁺, probably reflecting the
low-affinity ion binding sites involved in ion release to the extracellular side of the membrane. For both lipids, maximal activities were obtained at 65°C, which compares well with published studies of detergent-solubilized CopA by Mandal et al. (2002) and to our own unpublished observations of detergent-solubilized CopA.

**Atomic Model for CopA**

The new density map from DMPC/DOPE tubes was used as a template for building a model of CopA that revealed the juxtaposition of cytoplasmic domains and the organization of the transmembrane helices. The first step was to build an all-atom model for the core of CopA using an X-ray crystallographic structure of SERCA1a as a template. For the cytoplasmic domains, X-ray crystal structures for the isolated A domain of CopA (Protein Data Bank [PDB] ID code 2HC8) (Sazinsky et al., 2006a) and for the isolated pair of N and P domains of CopA (PDB ID codes 3A1C and 2B8E) (Sazinsky et al., 2006b; Tsuda and Toyoshima, 2009) were aligned with SERCA1a in the E2 conformation (PDB ID code 1IWO) (Toyoshima and Nomura, 2002). For the transmembrane helices and their connections to the cytoplasmic domains, the sequence of CopA was aligned with SERCA1a and used to model by homology. Two extra transmembrane helices are predicted in the N-terminal region of CopA and, after building the helices with the corresponding sequences (see Experimental Procedures), they were inserted between M1 and M2 of SERCA1a, as suggested by previous reports (Hatori et al., 2007; Lutsenko and Kaplan, 1995; Wu et al., 2006a).
et al., 2008). The resulting hybrid homology model lacked the N-terminal and C-terminal metal-binding domains, which have no counterpart in the SERCA1 template. This hybrid model was used for the docking studies described below.

The density map from DMPC/DOPE tubes was used to reposition the individual domains of this DC-CopA homology model to produce a fitted model. Specifically, after placing the homology model roughly into the envelope from the helical reconstruction, the loops connecting the domains were broken and the position of each cytoplasmic domain was refined using cross-correlation (using the Fit-in-Map feature of Chimera). The density map accounted well for these domains, with evidence for individual \( \alpha \) helices in several locations (Figure 5A; Movie S1). Surprisingly, the A, N, and P domains of CopA accounted for all of the densities in this region of the map, indicating that the NMBD was disordered. In our previous map from DOPC tubes, the NMBD was seen near the dimer interface, and its disordering in the current DMPC/DOPE tubes may be due to changes in this interface. Indeed, Figure 6 shows that there is an \( \sim 50^\circ \) angle between two-fold related molecules in the DMPC/DOPE tubes (defined by axes running from the middle of the transmembrane helices to catalytic aspartate), whereas these molecules were almost parallel in DOPC tubes. There is also a shift in the cytoplasmic domains relative to one another (Figure 7), which may explain the absence of the NMBD in the map from DMPC/DOPE tubes.

Protein densities were less distinct within the membrane than in the extramembranous regions of the map, although the general outline of the transmembrane domain could be discerned (Figure 5B). This loss of contrast is common in helical reconstructions and may be exacerbated in this case by the extra, disordered molecules hypothesized to face the outside of the tubes, which would contribute to the background density within the membrane. Despite the poorer definition of membrane domains, we were able to use our all-atom model of DC-CopA to interpret the transmembrane densities. Specifically, the six conserved transmembrane helices (M1–M2, M5–M8) fell within strong density. Similar to maps of SERCA from helical crystals (Xu et al., 2002; Young et al., 2001; Zhang et al., 1998), a density was observed at the cytoplasmic surface above M1 (arrowhead

Figure 4. ATPase Activity of Reconstituted DC-CopA
(A and B) Concentration dependence of activity for Cu⁺- and Ag⁺-dependent activity. A background level of \( \sim 0.3 \mu \text{mol/mg/min} \) has been subtracted from these data, which have been fitted with the Michaelis–Menten equation for a single binding site. Because there is inhibition at higher ion concentrations, the last two or three points of each curve have been omitted for this fit. The resulting values for apparent \( K_d \) and \( V_{\text{max}} \) are cited in the text.
(C and D) Temperature dependence of activity for Cu⁺ (C) and Ag⁺ (D) shows a peak at \( \sim 65^\circ \text{C} \), which is consistent with previous work with detergent-solubilized CopA. In all plots, the open circles represent DC-CopA reconstituted in DMPC/DOPE and the filled circles represent DC-CopA reconstituted in DOPC. Error bars correspond to the standard error of the mean.
in Figure 5A) that matches the bent extension of this helix seen in atomic structures of SERCA, Na+/K+-ATPase, and H+-ATPase. Additional membrane density was observed surrounding M4, which we fitted with the extra M2 and M3 helices that are characteristic of PIB ATPases. The location of these helices is slightly displaced from our previous model from DOPC tubes, with M2 taking the position of M3 in the previous map and M3 falling between M4 and M8. Given the poor contrast within the transmembrane domains, the precise location of these two extra helices remains tentative.

**Computational Docking of the NMBD**

Because our previous density map from the thinner DOPC tubes revealed the binding site of the NMBD next to the A domain, we were disappointed that the current map from DMPC/DOPE tubes did not provide further information at higher resolution. Nevertheless, we hypothesized that the NMBD binding site previously identified next to the A domain, although perturbed, still represented the most structurally compatible location for docking of the NMBD. To test this hypothesis, we performed in silico docking of the NMBD to the other cytoplasmic domains of CopA models obtained either by homology to the E2 state of SERCA or by fitting to our map from DMPC/DOPE tubes. This procedure uses Monte Carlo sampling to identify favorable docking sites on the molecular surface and ranks them energetically.

For our initial docking, we used the hybrid homology model of ΔNAC-CopA based on SERCA1 in the E2 conformation as a template, due to its close resemblance to the structure from DOPC tubes in which the NMBD was seen. For a search target, we made a homology model of NMBD based on the structure of a CopZ metallochaperone from Bacillus subtilis. This particular template was chosen for its high resolution (1.5 Å) and for its high sequence identity (41%) relative to the CopA NMBD from A. fulgidus. Although the CopZ template structure had Cu²⁺ bound to its CxxC motif, NMR structures of Cu⁺-free NMBD homologs indicated root-mean-square deviations of only ~1 Å, suggesting that structural changes induced by Cu⁺ binding are minimal. For docking, the homology models for Cu-free NMBD

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**Figure 5. Fitting an Atomic Model to the Map from DMPC/DOPE Tubes**

(A) Domains are shown in various shades of red and are labeled accordingly. Isolated densities for individual α helices are visible in A and P domains, and other α helices at the periphery of N and P domains fit snugly within the iso-surface density envelope. The envelope for the transmembrane domain (TM) shows a distinct bulge at the top right, consistent with the kinked cytoplasmic end of M1.

(B) Section through the transmembrane region of the density map overlaid with transmembrane helices, which are numbered. This is a view from the extracellular side of the membrane.

See also Movie S1.

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**Figure 6. Comparison of the ΔC-CopA Dimer Seen in the Two Helical Crystals**

(A) Dimer from the DMPC/DOPE tubes viewed parallel to the membrane surface. This view illustrates the >30° inclination of the transmembrane domain relative to the membrane normal.

(B) The dimer from DMPC/DOPE tubes viewed normal to the membrane surface.

(C) Dimer from our previous map from DOPC tubes (PDB ID code 2VOY; Wu et al., 2008) viewed parallel to the membrane surface showing that in this case the transmembrane domain is closely aligned to the membrane normal. The NMBD, which is visible in these crystals, is shaded in orange for the red monomer and purple for the blue monomer.

(D) Dimer from DOPC tubes viewed normal to the membrane surface. Note that (B) and (D) are at a smaller scale than (A) and (C); the transparent gray rectangles correspond to the membrane.
and CopA were treated as independent, unconstrained molecules free to rotate and translate in all directions in space, and the ICM docking routine evaluated numerous alternative binding interfaces, ultimately ranking them according to their predicted binding energy. Table 2 shows that the lowest-energy solution was well separated from the other solutions, making it the clearly preferred location for the NMBD on the molecular surface of CopA. Significantly, this binding pose is consistent with the location of the NMBD in DOPC tubes, despite the lack of physical constraints or any other information related to the EM structure. This docking result places the CxxC Cu-binding loop of the NMBD next to a conserved loop in the N domain that controls ATP binding, and when bound, the Cu+ ion would lie within 13 Å of the Mg2+ ion at the catalytic site of the P domain (Figure 7).

Unlike the model previously derived directly from the EM structure, the docking solution packed the two α helices of the NMBD against the A domain. Specific interactions at this interface also involve the loops between β strands composing the body of the A domain (residues 252–256, 270–274, 278–281, 295–298). The surface area buried in this interaction is 1943 Å², which is divided between the three domains as follows: 1040 Å² for the A domain, 507 Å² for the P domain, and 374 Å² for the N domain.

For comparison, we also used the fitted model corresponding to the conformation observed in DMPC/DOPE tubes as a template for docking, although in this case the apparent disordering of the NMBD in the corresponding crystals led us to expect a lower binding energy. Indeed, the ICM docking routine determined that the total energy for this pose was almost 17 kcal/mol higher for the DMPC/DOPE tube model compared to the SERCA1 homology model (−28.2 versus −44.9). These energy units are not absolute, due to inaccuracy in the force field and the coarseness of the grid on which potential energies were calculated, but are indicative of the relative binding strength. Accordingly, calculations of buried surface area showed a >40% decrease to 1176 Å². The reason for this reduced binding energy is evident in Figures 7C and 7D, where the homology model for CopA is overlaid with the fitted model for CopA from DMPC/DOPE tubes. This reveals a significant shift in the A and N domains relative to the P domain, and the loss of binding energy is consistent with an altered molecular surface for NMBD docking due to the disrupted interface between A, N, and P domains. Indeed, intermediate values for binding energy were obtained in docking studies with other permutations of the ΔNΔC-CopA model in which the N domain was either moved, modified, or removed altogether (data not shown). These results illustrate that all three cytoplasmic domains contribute to the NMBD binding site and that the binding was strongest when the conformation resembled the E2 state adopted by SERCA1.

Finally, to complete our model of ΔC-CopA, we constructed a linker between the M1 and the NMBD (Figure 7). In SERCA, two N-terminal α helices are attached to the front of the A domain and are connected to the cytoplasmic end of M1 via a flexible linker. Although the NMBD is associated with the side of the A domain in our model, the CopA linker sequence is long enough to wrap under the A domain and to connect to the kinked extension of M1. Thus, our model of ΔC-CopA is consistent with the idea that the physical couplings observed between SERCA transmembrane helices and the cytoplasmic domains are conserved in the Pib subfamily of pumps.
composed of DOPC. Although there were differences with our E2 conformation of SERCA1, the lowest-energy pose was mational determinants. Using a homology model based on the relative to the other CopA cytoplasmic domains and its confor- tational molecular docking to explore the site of NMBD binding precisely into the map. However, density for the NMBD is absent, of the three isolated cytoplasmic domains could be fitted into the map. Nevertheless, the transmembrane domain is strongly tilted relative to the DMPC/DOPE tubes reveals a distinct shift in N and A domains more, although it still appears to interact with residues in the DG(V/I)ND loop. In E2, the (T/S)GE loop has moved back and interacts with the conserved DG(V/I)ND loop at the periphery of the P domain. In our model fitted to DMPC/DOPE tubes, this (T/S)GE loop has moved back even more, although the latter configuration of cytoplasmic domains has not previously been seen in structures of P-type ATPases and could represent a transition from the E2 to the E1 state as the A domain begins to disengage from the P domain. The increased angle of the transmembrane domain relative to the P domain (Figures 7C and 7D) is consistent with this transition, as it eventually results in a 30° upward rotation of the P domain in the E1 state relative to the E2 state (Toyoshima and Nomura, 2002). Normally, the E2-to-E1 transition would be initiated by ions binding to their transport sites in the membrane. In the current situation, we speculate that a shearing of the transmembrane domain caused by its oblique angle relative to the membrane might be responsible for inducing this conformation.

Factors that may contribute to the unusually high tilt of the membrane domain are (1) the negative curvature of the crystal
contacts between cytoplasmic domains, (2) the intermolecular contacts that stabilize the dimer, and (3) the thinner bilayer produced by the shorter aliphatic chains of DMPC, which composes the bulk of the bilayer. Indeed, the hydrophobic core thickness of DMPC has been experimentally measured as 23 Å compared to 27 Å for DOPC (Lewis and Engelman, 1983), and this difference would be consistent with a tilt of ~30° in order to accommodate a hydrophobic domain with a fixed thickness (cos⁻¹[23/27]). Given the corresponding difference in the tilt of the CopA molecule in DMPC/DOPE versus DOPC tubes, it seems plausible that the thinner DMPC bilayer could be the driving force for altering the geometry of the dimer and thus producing the negative curvature of the crystal. Despite the dramatic effect on crystallization, however, the DMPC/DOPE bilayer had only modest effects on ATPase activities. It should be noted that the lipid-to-protein ratio was six times higher for this assay, where no crystalline arrays were observed to form. Nevertheless, the approximately 2-fold increase in $K_a$ and $V_{max}$ is comparable to the 2-fold changes in these parameters in detergent micelles (Rice et al., 2006) and can therefore be considered to represent the normal range of behavior for CopA in different hydrophobic environments. Furthermore, this range is in line with measurements of other P-type ATPases in lipid and detergent environments (e.g., Cornea and Thomas, 1994; Warren et al., 1974).

From a structural point of view, a shearing of the transmembrane domain would be required to maintain the upper and lower boundaries of the transmembrane helices as the domain was tilted in the bilayer. In other words, one would expect a relative movement of transmembrane helices along their helical axes. Such movements have in fact been described for the M1/M2 helical pair of SERCA, both during the transition from E2 to E1 (Toyoshima and Nomura, 2002) and during Ca<sup>2+</sup> occlusion by E1~P (Olesen et al., 2004). In the case of CopA, M2 and M3 are inserted between this M1/M2 pair and placed at the periphery of the transmembrane domain; accordingly, the entire M1–M4 bundle would likely slide relative to M5–M8, thus accommodating the shear force. By analogy with the structural changes during the E2-to-E1 transition in SERCA, this movement of M1–M4 would pull the A domain away from the P domain, potentially inducing the conformation seen in DMPC/DOPE tubes. A corresponding force on the NMDB, which is directly linked to M1, could also help disengage it from its binding site on the A domain. According to this hypothesis, M1 would no longer be tethered to the cytoplasmic domain in the E1 state. This implies that the more rigid connection between M4 (M2 in SERCA) and the A domain may be the important element that drives changes to the transmembrane domain and leads to ion occlusion during the transition to E1~P.

The site for the NMDB identified by in silico docking is consistent with the corresponding density in our previous map from DOPC tubes (Wu et al., 2008). The specifics of the interface between the NMDB and the A domain are different in our previous model, presumably reflecting our earlier neglect of binding energy and molecular surface compatibility during manual assembly and fitting. Nevertheless, in both cases, the NMDB binds to the side of the A domain with additional interactions between the GMTCAMC<sup>30</sup> Cu-binding loop and the conserved ERRSEHP<sup>463</sup> loop on the N domain. This ERRSEHP<sup>463</sup> loop has been shown in NMR and X-ray crystal structures of isolated domains to be intimately involved in ATP binding (Banci et al., 2010; Dmitriev et al., 2008). In particular, His462 makes critical interactions with the α- and β-phosphates and with the adenine ring of ATP (Szakácsy et al., 2006b; Tsuda and Toyoshima, 2009); its mutation in ATP7A almost completely prevents ATP binding (Morgan et al., 2004) and the H462Q mutation is the most common cause of Wilson’s disease (Thomas et al., 1995). Interaction between the NMDB and the N domain has been demonstrated experimentally by Gonzalez-Guerrero et al. (2009) using pull-down assays. Specifically, they showed that Cu-free NMDB bound to an N/P-domain construct whereas Cu-loaded NMDB did not. Furthermore, these investigators showed that interaction between the Cu-free NMDB and the N/P-domain construct was disrupted by ADP-Mg<sup>2+</sup>. Although our docking result indicates that the majority of binding energy for NMDB comes from its interface with the A domain, the interface between the NMDB and the N and P domains still accounts for 891 Å<sup>2</sup> of buried surface (out of a total of 1934 Å<sup>2</sup>), which seems sufficient to explain the pull-down results. It is interesting to note that the altered conformation represented in our DMPC/DOPE crystals results in a loss of 767 Å<sup>2</sup> of buried surface (i.e., almost the entire interaction with the N and P domains). This observation, together with the disordering of the NMDB in these crystals, suggests that the interaction with the A domain alone is not enough to retain NMDB at its binding site and that its association with the cytoplasmic domains is transient.

This transience is also implied by interaction between the NMDB and metallochaperones. Physiologically relevant metallochaperones have been shown to transfer Cu<sup>2+</sup> to MBDs of both ATP7A (Walker et al., 2002, 2004) and CopA (Gonzalez-Guerrero and Argüello, 2008), and NMR structures of the complex between the HA1 metallochaperone and related MBDs of ATP7A indicate that the α-helical face of NMDB interacts with the corresponding face of the metallochaperone during Cu<sup>2+</sup> transfer (Achila et al., 2006; Banci et al., 2005a, 2005b). According to our model, these α helices are packed against the A domain, implying that the NMDB must dissociate prior to interacting with metallochaperones. This dissociation step may be the kinetic barrier to activation that has been revealed by kinetic analysis of the transport cycle (Hatori et al., 2009). An alternative binding site for the NMDB on the opposite side of the A domain, where it would not have interactions with either the N or P domain, has been proposed on the basis of chemical crosslinking (Lübchen et al., 2009). This alternative is not compatible with our previous density map or with the results of molecular docking, and it is difficult to explain how the NMDB would play an autoinhibitory role from that location.

There is a growing consensus that NMDBs play a regulatory role for Cu pumps and are not involved in transferring Cu<sup>2+</sup> to the transport sites within the transmembrane domain (Argüello et al., 2007). This conclusion applies both to the human pumps (ATP7A and ATP7B), which carry six MBDs on their N terminus, and to CopA from bacteria, which generally have a single N-terminal MBD. In particular, several studies have shown Cu transfer from metallochaperones to MBDs (Achila et al., 2006; Banci et al., 2005a, 2005b; González-Guerrero and Argüello, 2008; Larin et al., 1999; Ralle et al., 2004; Singleton et al., 2009; Strausak et al., 2003) and from metallochaperones to
transport sites (González-Guerrero et al., 2009). However, disruption of MBDs by either truncation or mutation does not affect transport activity of bacterial pumps (Bal et al., 2001; Fan and Rosen, 2002; Mana-Capelli et al., 2003; Mandal and Argüello, 2003; Mitra and Sharma, 2001; Rice et al., 2006), and disruption of MBD on human pumps mainly affects enzyme activation and trafficking (Cater et al., 2007; Huster and Lutsenko, 2003; Strausak et al., 1999; Tsivkovskii et al., 2001; Veldhuis et al., 2009b; Walker et al., 2002). An enzymatic study of CopA from Thermotoga maritima concluded that the NMBD was responsible for kinetic inhibition of the reaction cycle and that this inhibition was alleviated by its binding of Cu⁺ (Hatori et al., 2008). Our structure offers a structural explanation for this auto-inhibition. Specifically, the NMBD has substantial interactions with both the A and N domains in our hybrid model of the E2 conformation. Proteolytic digestion patterns of CopA indicate that these domains undergo large movements during the transition from the E2 state to the E1–P state, similar to SERCA (Hatori et al., 2009), and the binding energy of the NMBD would resist these movements. Cu⁺ could have a role in disrupting this interaction in three ways: (1) by binding to transport sites and driving a conformational change; (2) by binding directly to the GMTCAMC loop at the interface between the NMBD and the N domain; and (3) by promoting interaction between the NMBD and metallochaperones, which would require dissociation of the NMBD from the A domain in order to form the corresponding complex.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification

Plasmids containing the C-terminal truncation of the copA gene (Wu et al., 2008) behind a pbAD promoter were expressed in E. coli (strain LMG1940) using LB broth with 0.1 mg/ml ampicillin at 37°C. After allowing 1 cultures to reach an OD of 1, expression of CopA was induced with 0.02% arabinose for 2 hr at 30°C. Cells were then harvested by centrifugation at 10,000 x g for 10 min and the pellet was suspended in 50 mM Tris-Cl (pH 7.5), 20% glycerol, 50 mM NaCl, 2 mM β-mercaptoethanol, and protease inhibitor cocktail (Hoffmann-La Roche). Cells were broken by French press at 20,000 psi and, after removing large debris by centrifugation at 10,000 x g for 20 min, membranes were collected by centrifugation at 150,000 x g for 1 hr and stored at −80°C. Membrane pellets were solubilized in 10 mg/ml n-dodecylβ-D-maltoside (DDM; Anatrace), 25 mM Tris-Cl (pH 7.5), 100 mM NaCl, 20 mM imidazole, 10% glycerol, 2 mM β-mercaptoethanol, and protease inhibitor cocktail at a protein concentration of 10 mg/ml. After gentle mixing for 30 min, insoluble material was removed by centrifugation at 150,000 x g for 30 min and the supernatant was loaded onto a 5 ml HiTrap Chelating HP column (GE Healthcare), which was charged with 0.1 M NiCl₂ and equilibrated with 25 mM Tris-Cl (pH 7.5), 100 mM NaCl, 20 mM imidazole, 10% glycerol, 2 mM β-mercaptoethanol, and 0.1% DDM. After washing, purified ΔC-CopA was eluted with 25 mM Tris-Cl (pH 7.5), 100 mM NaCl, 400 mM imidazole, 10% glycerol, 2 mM β-mercaptoethanol, and 0.1% DDM. Overnight incubation with thrombin (1 U/mg of protein) was used to remove the polyhistidine tag. A benzamidine-Sepharose column (Amersham Biosciences) equilibrated with 25 mM Tris-Cl (pH 7.5), 100 mM NaCl, 10% glycerol, 2 mM β-mercaptoethanol, and 0.1% DDM was used to remove the thrombin. Finally, purified ΔC-CopA protein was dialyzed against 25 mM Tris-Cl (pH 7.5), 100 mM Na₂SO₄, 10% glycerol, 2 mM DTT, and 0.1% DDM. All the steps in this purification were done at 4°C.

ATPase Activity Assays

For measuring ATPase activity, proteoliposomes were made with a 2.5:1 lipid:protein weight ratio using either DOPC or a 4:1 weight ratio of DMPC/DOPE. To make these proteoliposomes, ΔC-CopA (solubilized in DDM) and the relevant lipid were dialyzed against 50 mM Tris-SO₄ (pH 7.5), 50 mM Na₂SO₄, 50 mM K₂SO₄, 10 mM MgSO₄, and 2 mM β-mercaptoethanol for 5 days at 4°C. As described in our previous work on CopA (Rice et al., 2006), the malachite green assay (Kimura et al., 1996) was used to quantitate phosphate production in 50 mM Tris-SO₄, 200 mM Na₂SO₄, 3 mM MgSO₄, 5 mM glutathione, 2.4 mM ATP, 10% glycerol, and 0.025% NaH₂PO₄. To test dependence on either Cu⁺ or Ag⁺, assays were performed at 70°C; to test temperature dependence, assays were performed with either 2 μM Cu⁺ or 1 μM Ag⁺. In order to prevent buildup of transport ions inside the vesicles, a small, subsolubilizing amount of detergent (0.01% DDM) was added to make the vesicles leaky. All nonreducing buffers were pretreated with 100 mg/ml Chelex (Bio-Rad) for at least 1 hr. Aliquots of ΔC-CopA proteoliposomes at 0.02 and 0.04 mg/ml were prepared and stored on ice, where the enzyme is completely inactive. Using a programmable PGR thermocycler (Eppendorf North America), duplicate 45 μl aliquots of the reaction mixture were heated to 70°C for 3, 5, 7, and 9 min and then cooled to 4°C to stop the reaction. Phosphate was then quantified in 96-well microtiter plates using a development time of 3 min; absorbance was read at 630 nm. In order to maintain a pH of 6.3 at a range of different temperatures, the following buffers were adjusted to the corresponding pHs at room temperature: MES-SO₄ (pH 6.2) for 15°C, MES-SO₄ (pH 6.3) for 25°C, MES-SO₄ (pH 6.4) for 35°C, MES-SO₄ (pH 6.5) for 45°C, MES-SO₄ (pH 6.6) for 55°C, MOPS-SO₄ (pH 6.9) for 65°C, Tris-SO₄ (pH 7.7) for 75°C, Tris-SO₄ (pH 8) for 85°C, and Tris-SO₄ (pH 8.3) for 95°C. Like in previous work, we observed a background of 0.3–0.35 μmol/mg/min in the absence of added Cu⁺ or Ag⁺; this background activity was abolished by the Cu chelator BCDs, suggesting that it arises from trace Cu⁺ in the solutions. After subtracting the background activity, the data were fitted with the Michaelis–Menten binding model to determine the apparent dissociation constant (Kₘ).

Cryocrystallography and Cryo-Electron Microscopy

ΔC-CopA tubular crystals were grown with a 4:1 mixture of DMPC/DOPE at a protein concentration of 1 mg/ml and at a lipid:protein weight ratio of 0.4. Dialysis was carried out for 5 days in 50 μl dialysis buttons (Hampton Research) at 30°C against 500 ml of 50 mM MES (pH 6.1), 25 mM Na₂SO₄, 25 mM K₂SO₄, 200 mM BCDS, 10 mM MgSO₄, and 2 mM β-mercaptoethanol. Stock solutions of lipid for both crystalization and ATPase assays were made in dodecyl octa-ethylene glycol ether (C₁₂E₈) at 1 mg lipid per 2 mg detergent. For cryo-EM, a suspension of crystals was deposited on a holey carbon grid and rapidly frozen in liquid ethane. Samples were imaged at −175°C with a CT3500 cryoholder (Gatan) at 50,000× nominal magnification with a CM200 FEI electron microscope (FEI) operating at 200 kV. Electron micrographs were recorded on Kodak SO-163 film and, after screening by optical diffraction, suitable images were digitized at 14 μm intervals with a Zeiss SCAI microdensitometer (Integraph).

Helical Reconstruction

Tubular crystals with a diameter of ~600 Å, displaying symmetric layer lines by optical diffraction and with defocus values between 1.5 and 2.5 μm, were selected for helical processing using Fourier-Bessel methods (Díaz et al., 2010). Layer lines were cataloged with a consistent set of Miller indices, which reflect the underlying 2D lattice within the membrane. To define the helical symmetry, Bessel orders were assigned to all of these layer lines (Figure 1). Initial assignments were based on phase relationships across the meridian, the radial location of the diffraction maximum, and the self-consistency of assignments across the transform (e.g., the construction of a plausible n,1,plot). To verify the Bessel order assignments, several alternative indexing schemes were tested, using EMPI helical reconstruction software (http://cryoem.nysbc.org/EMPI-helm) to compare phase residuals for the determination of out-of-plane tilt (q). For highly tilted tubules (q~5°), this phase residual was definitive in identifying the correct indexing. Near-φ/2-phase residuals and two-fold phase residuals were also consistently lower with the correct indexing. In the end, EMPI was used to apply the unbending methods of Beroukhim and Unwin (1997) to 12 tubes. Correction for the contrast transfer function was based on an amplitude contrast of 7% (Toyoshima et al., 1993) and the resolution of the final maps was truncated at 9 Å resolution, where the two-fold related phase residual approached 43° (random data produce a 45° residual).
Modeling and Docking

All modeling and docking procedures were carried out using the ICM protein docking software (v3.6-1f; Abagyan and Totrov, 1994; Cardozo et al., 1995) as previously described (Allen and Stokes, 2011). Briefly, the sequences of CopA and SERCA1α were aligned by Needleman and Wunsch global alignment using zero end-gap penalties (Abagyan and Batalov, 1997). A 3D model of CopA (omitting N- and C-terminal metal-binding domains) was then constructed by threading its sequence onto the X-ray crystallographic structure for SERCA1α in the E2 conformation (PDB id code 1WOC) using the alignment as a guide. For this homology model, the geometries of conserved residues were fixed, whereas the nonconserved residues were set to their standard geometries and a local minimization was performed to relieve any clashes. Structural databases were consulted for probable configurations of the non-conserved loops or, in the event no reasonable solution was found, the loop was modeled freely to its lowest-energy conformation (Cardozo et al., 1995).

Existing X-ray crystal structures of CopA cytoplasmic domains (the isolated P/N-domain pair, PDB id code 3A1C, and the isolated A domain, PDB id code 2HCB) were then aligned with the initial homology model, and this model was adjusted to exactly match the X-ray structures in overlapping regions. Thus, the resulting hybrid homology model of CopA inherits X-ray crystallographic information from both the SERCA1α E2 conformation and the isolated structures of the CopA cytoplasmic domains. A homology model for the NMBD of CopA based on the X-ray crystallographic structure of the metallochaperone CopZ (PDB id code 2QIF) was built in ICM using the same approach as above. Finally, we used Coot (Emsley et al., 2010) to make minor adjustments or refinements to the model and Phyre (Bennett-Lovsey et al., 2008) to build the extra M2 and M3 helices of CopA, and then several rounds of energy minimization were performed in ICM. Finally, Chimera (Petterson et al., 2004) was used to visualize results and to make figures.

After building these atomic models, the NMBD was docked to the hybrid model of CopA (N, P, and A domains) using ICM protein-protein docking (Fernandez-Reco et al., 2005). ICM first predicted surface patches on the cytoplasmic domains that were likely to produce energetically favorable protein interactions using the optimal docking area method. Next, ICM computed electrostatic, van der Waals, hydrogen-bond, solvation, and hydrophobic potentials on a coarse grid over these surface regions. Interaction energies were determined for a Monte Carlo sampling of NMBD poses at each of the surface patches. The 400 most favorable results were then subjected to side-chain energy minimization and ranked according to their overall binding energy. Buried surface area was determined in CNS (Brunger, 2007) with a 1.4 Å probe radius.

ACCESSION NUMBERS

Coordinates for the two models have been deposited in the Protein Data Bank under ID codes 3J08 and 3J09, and the map has been deposited in the Electron Microscopy Databank under accession number 5271.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and one movie and can be found with this article online at doi:10.1016/j.str.2011.05.014.

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