Structural basis of ion uptake in copper-transporting P1B-type ATPases

Salustros, Nina; Gronberg, Christina; Abeyrathna, Nisansala S.; Lyu, Pin; Oradd, Fredrik; Wang, Kaituo; Andersson, Magnus; Meloni, Gabriele; Gourdon, Pontus

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Copper is essential for living cells, yet toxic at elevated concentrations. Class IB P-type (P_{1B}) ATPases are present in all kingdoms of life, facilitating cellular export of transition metals including copper. P-type ATPases follow an alternating access mechanism, with inward-facing E1 and outward-facing E2 conformations. Nevertheless, no structural information on E1 states is available for P_{1B}-ATPases, hampering mechanistic understanding. Here, we present structures that reach 2.7 Å resolution of a copper-specific P_{1B}-ATPase in an E1 conformation, with complementing data and analyses. Our efforts reveal a domain arrangement that generates space for interaction with ion donating chaperones, and suggest a direct Cu\(^+\) transfer to the transmembrane core. A methionine serves a key role by assisting the release of the chaperone-bound ion and forming a cargo entry site together with the cysteines of the CPC signature motif. Collectively, the findings provide insights into P_{1B}-mediated transport, likely applicable also to human P_{1B}-members.
extracellular/luminal (out) sides (Fig. 1)\textsuperscript{14,15}. Cargo uptake and occlusion from the cytosol is accomplished in E1 conformations, and together with ATP-dependent phosphorylation of an invariant, catalytic aspartate in the P-domain yielding the E1P form, this leads to the transition to the outward-facing E2P state. The cargo is then released to the extracellular space, and upon dephosphorylation (E2), the pump returns to the inward-facing E1 conformation.

Thus far, structural information on the P\textsubscript{in}-ATPase core is limited to structures of CopA from Legionella pneumophila (LpCopA), ATP7B from Xenopus tropicalis, and ZnT4 from Shigella sonnei in E2 and E2P conformations\textsuperscript{20,21}, as well as individual soluble domains and a low-resolution cryo-EM model of CopA from Archaeoglobus fulgidus (AfCopA)\textsuperscript{22–24}. Consequently, no metal-bound (E1) conformation of a P\textsubscript{in}-ATPase has been reported, leaving basic mechanistic principles regarding ion uptake, transfer and binding elusive. Typically, copper-proteins deliver copper to P\textsubscript{in}-ATPases in vivo, i.e., Archaeoglobus fulgidus CopZ to AfCopA\textsuperscript{25} or homologous Atox1 to human ATP7A/B. However, although it has been proposed that an electropositive platform, MB\textsubscript{in}–in between MB and MI is involved in the recruitment of CopZ, the molecular determinants of chaperone-mediated Cu\textsuperscript{2+} transfer to the ATPase are elusive. Here, we report three inward-facing EI structures of a copper-transporting P-type ATPase, providing fundamental insights into ion uptake and transport by the entire P\textsubscript{in}\textsubscript{–}subclass.

Results and Discussion

Structure determination

To shed further light on the ion entry and transport mechanism of P\textsubscript{in}-ATPases, we targeted CopA from the model organism Archaeoglobus fulgidus using a truncated construct lacking both the N- and C-terminal heavy metal binding domain (AfCopA\textsubscript{ΔNΔC}). Crystallization was conducted in the presence of high concentrations of lipid and detergent (the HILId method\textsuperscript{26}) and 0.5 mM CuSO\textsubscript{4}, which yielded crystals diffracting to 2.7 Å resolution. The structure was determined using molecular replacement as a phasing method. The final model yielded R/Rfree of 22.5/25.7 (Supplementary Table 1) and enabled modeling of side chains in the A-, P- and most parts of the M-domain, whereas somewhat lower local resolution was observed in the N-domain, likely due to the absence of nucleotide and the lack of crystal contacts in that region (Supplementary Figs. 2–3 & Methods).

A unique EI conformation

The recovered AfCopA structure exhibits the anticipated P\textsubscript{in}-ATPase architecture\textsuperscript{19,21,26}, including the cytosolic A-, P- and N-domains and a total of eight membrane-spanning helices, MA, MB and MI-M6, clearly separating these molecular pumps from other types of P-type ATPases (Fig. 1a & Supplementary Fig. 1). However, the nucleotide-binding pocket of the N-domain is facing the catalytic aspartate (D424) in the P-domain, while there is a large distance between the A- and N-domains, clearly deviating from previously determined conformations of P\textsubscript{in}-ATPases. To integrate the determined AfCopA structure into the overall P-type ATPase reaction cycle, we performed structure-based alignments of the soluble domains to the available structures of the well-studied Ca\textsuperscript{2+}-transporting sarco/endoplasmic reticulum P-type ATPase (SERCA), revealing highest similarities to [Ca\textsuperscript{2+}]\textsubscript{1}E1-ATP conformations (PDB-IDs 3N8G, 1T5S & 1T5T) (Supplementary Fig. 4). This was unexpected, as the AfCopA crystals were generated in the presence of cargo, but without ATP or non-hydrolysable analogues thereof, i.e., equivalent to conditions that capture early EI states of SERCA. In addition, crystals of AfCopA obtained in the absence of copper and nucleotide yielded a structure in a similar overall conformation, again indicative of an early EI conformation and perhaps an EI (ground state) preference for this particular CopA member (Supplementary Figs. 3–5 & Methods).

However, the positions of the conserved dephosphorylation (T/S)GE and nucleotide binding (HP) motifs of the A- and N-domains, respectively, relative to the P-domain in the determined AfCopA structure do not reveal similarities to any of the available structures of SERCA (Fig. 1 & Supplementary Figs. 4 & 6). This contrasts to the previously reported E2P and E2.P\textsubscript{i} structures of P\textsubscript{in}-ATPases, which superpose well with the corresponding SERCA conformations (Fig. 1b & Supplementary Fig. 4). The peculiar arrangement of the AfCopA soluble domains in the determined EI state relative to SERCA becomes particularly clear by considering the position of the invariant (T/S)GE dephosphorylation loop of the A-domain. In the AfCopA structure, it is placed at the end of M4, while it is located on the other side of the P-domain in EI structures of SERCA (PDB-IDs 4H1W & 3N8G)\textsuperscript{27,28} (Fig. 1b & Supplementary Fig. 7).

Noteworthy, the configuration of the AfCopA soluble domains rather resembles the recently determined EI and EIP states of the Class 1A P-type ATPase KdpB, which is part of the KdpFABC complex\textsuperscript{29,30} (Supplementary Fig. 7). The observed similarities between P\textsubscript{in}\textsubscript{A} and P\textsubscript{in}\textsubscript{P}ATPases can be rationalized by the shared evolutionary origin and alike topology of the subfamilies, i.e., the absence of an N-terminal A-domain extension and helices M8–10, which are present in other classes of P-type ATPases. However, the integration of KdpB into the KdpFABC complex makes it atypical member of the P-type ATPase superfamily, and P\textsubscript{in}-ATPases likely operate in a profoundly different manner. We therefore conclude that the determined AfCopA structure resembles a deviant EI conformation, likely affecting the P\textsubscript{in}-ATPase mode of action.

The A-domain modulates the catalytic cycle

Structural comparisons to the previously determined E2P, structure of LpCopA\textsuperscript{31} disclose major rearrangements during the E2 → EI transition (Fig. 2 & Supplementary Fig. 8). Most noticeably, a large A-domain rotation of approximately 100° relative to the P-domain moves the conserved (T/S)GE dephosphorylation motif from the catalytic aspartate of the P-domain, positioning it immediately in the vicinity of the end of M4, where it is stabilized by electrostatic interactions with the P-domain (Supplementary Fig. 9). The CopA A-domain and (T/S)GE motif undergo a counterclockwise rotation relative to the P-domain as seen from the cytosol. Along with the displacement of the A-domain, the N-domain is tilted, thereby closing the cytosolic headpiece, priming the nucleotide-binding pocket for ATP binding (Supplementary Fig. 10).

The E2 → EI counterclockwise rotation of the A-domain is similar to the situation in KdpB\textsuperscript{32} but opposite to SERCA, where the TGE motif rotates clockwise (Supplementary Fig. 7). But what is the mechanistic consequence and origin of this fundamental difference between P\textsubscript{2} and P\textsubscript{2.P}\textsubscript{i}-ATPases? The distinct location of the A-domain in CopA and SERCA must be dictated by the overall architecture. As indicated above, P\textsubscript{in}-ATPases lack the N-terminal A-domain extension and, consequently, the A/MI-linker. In SERCA, this connecting stretch determines the interspace between M1 and the distal portion of the A-domain, leaving the distance essentially constant in-between the E2.P\textsubscript{i} and EI states (Supplementary Fig. 11). This is of particular importance as the length of the A/MI-linker is known to be critical for the SERCA reaction cycle, with insertions or deletions abolishing ATPase activity\textsuperscript{32}. Conversely, due to the absence of the N-terminal A-domain extension in P\textsubscript{in}-ATPases, the A-domain is less conformationally restricted, and the aforesaid distance decreases from 69 to 65 Å during the E2.P\textsubscript{i} → EI transition (Supplementary Fig. 11).

Analogously, modifications of the A/M3 linker, which generally adopts an elongated loose shape in EI states while it is more structured in E2 conformations, affects the function of SERCA\textsuperscript{33}. This transport cycle shift in SERCA is likely supported by stabilizing electrostatic interactions between the A- and P-domains in the E2 conformations\textsuperscript{34}. However, the parts of the P- and A-domains forming these interactions
are absent in P1B-ATPases (Supplementary Fig. 9). Lack of these features confers an overall electronegative surface patch to the P-domain of CopA, which permits interaction with electropositive residues of the A-domain also in the determined E1 conformation (Supplementary Fig. 9). As a consequence, the A/M3 linker is shortened by 4.6 Å with the E2.Pi→E1 transition, while the distance is enlarged with 3.3 Å in SERCA (Supplementary Fig. 11).

Collectively, this demonstrates that the push-and-pull forces exerted on the A-domain during the course of the transport mechanism are significantly different in-between P1- and classical P2-ATPases, in agreement with the detected atypical arrangement of the A-domain of CopA. It is possible to speculate that the postulated absence of counterions and structural differences such as the missing A/M1 linker in P1B-ATPases leads to the need for alternative stimulation of the E2.Pi→E1 transition. The tighter restraints of the A/M1 and A/M3 linkers in the E1 state may serve such as purpose, powering the A-domain rotation required for reaching the E1 conformation.

Copper entry to the transport pathway - conserved residues in the M-domain become accessible from the cytoplasmic side

Unexpectedly, in the M-domain, MA-M2 and M3-M6 each form a helix bundle, which move relative to one another in the E2.Pi→E1 transition, as revealed by unbiased analyses of the relative locations of each transmembrane helix in the two states (Fig. 2 & Supplementary Fig. 12). In the determined E1 structure, M2 and M6 converge in the intersection of these sub-domains. As a consequence, the previously proposed ion exit pathway becomes obstructed, rendering it inaccessible from the outside as apparent from analysis using the software HOLLOW (Fig. 2a–d). In contrast, on the opposite side, the protein interior is exposed to the intracellular environment. Thus, the determined structure displays an inward-facing E1 conformation.

Notwithstanding the absence of structural information, copper binding to P1B-ATPases has been extensively studied. Based on the Pearson acid-base (HSAB) theory, it is anticipated that Cu+ with a relatively small charge and "soft" nature will more favorably be coordinated by "softer" and more polarizable cysteines, methionines and to a lesser extent histidines in contrast to other metal ions. Accordingly, ion uptake from the cytosol has been proposed to occur via an entry site formed by the conserved M158 (at the MB’-M1 transition), E205 (M2) and D336 (M3). An alternative sulfur-based model was introduced later, in which Cu+ binds to a transient site formed by M158 together with C380 and C382 of the CPC motif located at a characteristic kink of M4. The ion would then be shuttled from the entry site or/via the transient site to the high-affinity ligands in the M-domain. High-affinity ion binding is expected between transmembrane helices M4-M6, harboring several invariant amino acids,
including the CPC residues as well as Y682, N683, M711, S714, and S715 of the YN (M5) and MxxSS (M6) motifs.

In the determined AfCopA structure, unlike the E2P and E2.Pi conformations of LpCopA, C380 and C382 are surface exposed, oriented towards M1-2 including M158 in the final model, again indicative of an early E1 conformation congruent with ion uptake (Figs. 2a & 3a). However, while the arrangement of M158, C380, and C382 is in agreement with Cu\(^+\) coordination in conjunction with the above-mentioned transient site, we were unable to unambiguously assign metal presence in the high-resolution data set (Fig. 4d). Conversely, Y682, N683, and M711 are facing the center of M4-6, primed for subsequent high-affinity Cu\(^+\) binding, hence including a substantial displacement of M711 relative to the E2.Pi conformation where it is exposed to the surrounding membrane (Fig. 3d). To investigate the functional significance of these conserved residues on AfCopA-mediated copper transport, we performed in vitro ATPase activity measurements on protein reconstituted into liposomes (Methods). The determined Cu\(^+\) -stimulated turnover of wild type AfCopA\(_{\Delta N \Delta C}\) was 242 ± 26 nmol P\(_i\) mg\(^{-1}\) min\(^{-1}\). Alanine substitutions of the M4-6 residues abolished the function while less dramatic effects were detected for the amino acids previously proposed to be involved in ion uptake (Fig. 3a), which is in agreement with earlier observations on CopA proteins.

Alanine substitutions of the M4-6 residues abolished the function while less dramatic effects were detected for the amino acids previously proposed to be involved in ion uptake (Fig. 3a), which is in agreement with earlier observations on CopA proteins. In addition, we measured Cu\(^+\) binding to AfCopA\(_{\Delta N \Delta C}\) and selected alanine substitutions by Inductively Coupled Plasma - Mass Spectrometry (ICP-MS), revealing an average stoichiometry of 0.87 ± 0.10 Cu\(^+\) ions per AfCopA\(_{\Delta N \Delta C}\) protein (Fig. 3b). Cu\(^+\) binding was particularly impaired by mutation of the cysteine residues C380 and C382 in the conserved CPC motif in M4, but also the YN (M5) and MxxSS (M6) motifs (Fig. 3b). Therefore, our data support the requirement of numerous conserved residues in M4-6 for CopA-mediated Cu\(^+\) transport, while the orientation of these side chains in the determined structure suggests an early E1 conformation, as
Despite the open conformational flexibility around the Cu+ entry site for copper uptake, in agreement with the isolated early E1 structure of AfCopA as observed from the cytoplasmic side of the relevant residues in the entry region c and putative high-affinity ion binding region d with the final electron density at σ = 1 shown in blue mesh.

![Diagram](image1)

**Fig. 3 | Functional characterization.** a Cu+ stimulated ATPase activity of selected mutant forms as determined by a colorimetric in vitro ATPase assay based on protein reconstituted in liposomes. WT wild type AfCopAAΔNΔC. Data were collected on material from two independent purifications with n = 5 (M158A, E205A, D336A, C382A, S714A, S715A, D426N) or n = 6 (other mutants), and are presented as mean ± SD. Source data are provided as a Source Data file. c, d Close-views of the AfCopA E1 structure as observed from the cytoplasmic side of the relevant residues in the entry region c and putative high-affinity ion binding region d with the final electron density at σ = 1 shown in blue mesh.

Achieved by reorientation of two bundles of helices within the M-domain.

**Cu+ binding to, and conformational flexibility around, a transient entry site**

The number and location of the high-affinity Cu+ binding sites in Pim1 ATPases is debated, representing a key unresolved issue in the field. Despite the open configuration of our structure and with the intention to further study the details of the physico-chemical environment of the transient site, we collected X-ray crystallographic data at the copper absorption edge (1.37 Å) diffracting to 3.3 Å. MR-SAD phasing in phenix.autosol14 using the final AfCopA E1 structure as a search model resulted in a substructure with indications of a single Cu+ site close to C382 of the CPC motif, without sign of copper between M4-6, although it cannot be excluded that the signal relates to a non-copper atom (Fig. 3). As such, the identified site resembles a flexible, transient Cu+ entry site for copper uptake, in agreement with the isolated early E1 conformation. Indeed, two complementing 100 ns MD simulations with a Cu+ initially placed in the trigonal sulfur position of AfCopA, indicate rapid transfer towards M711 together with two or three coordinating water molecules (Supplementary Fig. 13).

![Diagram](image2)
CopZ-mediated Cu⁺ delivery

How is Cu⁺ uptake from the milieu then accomplished in PₐT-ATPases? Noticeably, the determined AFcopA structure features low electronegativity in the Cu⁺ uptake region surrounding M158, C380 and C382. Investigation of single ensembles reveals that only minor rearrangements around the Cu⁺ entry site strongly affect the electrostatic surface (Fig. 4a). In particular M158 has a strong effect on the charge exposed to the surroundings. Instead, the presence of a coordination environment rich in sulfur-containing ligands (such as methionines and/or cysteines) as observed here may drive the Cu⁺ transfer event.

In this context, it is also of importance that in contrast to other ions, cystolic Cu⁺ is at all times bound to chaperones, and according to the current model, expected to be transferred directly from these small soluble proteins such as CopZ to CopA²⁴. Certain of the conformations of M158 detected by our ensemble refinement approach are positioned within 6 Å of the CopZ-bound Cu⁺-ion (Fig. 4c), illustrating that direct Cu⁺ transfer from CopZ to M158 may well be possible. Such a direct transfer of Cu⁺ would require the presence of yet another transition site, in which M158 is involved facing the cytosol, together with the CxxC cysteines of CopZ (Fig. 4). Cu⁺ would then be handed over to the entry site formed by M158, C380 and C382, and the membranous high-affinity binding site(s). We speculate that the surface exposed E205 and D336 are involved in Cu⁺ uptake by creating a suitable environment to stimulate release from CopZ, or through stabilization of the CopA·CopZ complex, unifying the available mechanisms of copper uptake.

Previous biochemical studies comparing the in vitro ATPase activity of wild type CopA stimulated by free (using cysteine as a vehicle) Cu⁺ or Cu⁺-loaded CopZ revealed that only the Cu⁺-CopZ-stimulated ATPase activity was abolished through alanine substitutions in the entry area, while free Cu⁺-stimulated ATPase activity remained unchanged³³. This not only agrees with our data on AFcopAANAC (Fig. 3a), but also emphasizes the specific role of M158, E205 and D336 in ion uptake. Since Cu⁺ is delivered to the ATPase core by chaperones, these residues in the entry area seem to be important in vivo, likely because they generate an appropriate docking and transfer region for Cu⁺ release from the chaperones combined with steric inability of the chaperones to directly reach the membranous high-affinity Cu⁺ binding ligands. Cysteine-bound Cu⁺ may well evade this requirement, replacing M158 as donors to the CPC cysteines. Thus, our E1 structure also provides a plausible Cu⁺ donation hypothesis to CopA proteins, combining the previous, somewhat conflicting models of ion uptake.

Transport and regulation mechanism

Based on detailed analyses of the structurally determined E1 state and comparisons to already available structures combined with functional characterization, we propose that copper specific PₐT-ATPases operate using the following transport mechanism (Fig. 5). The E₂.Pᵢ → E₁ transition occurs along with major conformational changes, in particular a large rotation of the A-domain, likely triggered through phosphate release upon dephosphorylation in agreement with the conserved P-type ATPase alternating access mechanism. In conjunction, M₄-M₂ shift relative to M₃-6, exposing the Pₐ₄ type ATPase-specific CPC motif of M₄ to the cytoplasm (Figs. 2a & 4).

The E₁ structure represents a Cu⁺ uptake state capable of binding to cargo-bound CopZ, as confirmed by computational docking. Alternatively, Cu⁺ may be provided to the ATPase core by the N-terminal HMBD, which is structurally homologous to CopZ, or small molecular ligands such as glutathione. The unique position of the A-domain, that is different to P₂- and P₃-ATPases, is required to create sufficient space for CopZ docking to the ATPase core, as its position in SERCA would sterically hinder the interaction. Orientation of the M₁₅₈ side chain towards the cytosol, as determined using the ensemble refinement approach, then allows trigonal-planar Cu⁺ coordination together with the cysteines of the conserved CxxC motif of CopZ, i.e. a transient site formed by mixed CopA-CopZ residues without direct influence of E205 and D336 (Fig. 4), in agreement with the sulfur-based transport pathway previously proposed for LpCopA³⁶. However, it cannot be excluded that E205 and D336 directly participate in the ion transfer, too. In the determined E₁ state, the side chain conformations of M158, C380 and C382 allow for trigonal-planar Cu⁺ coordination (Figs. 3b & 4). This is supported by anomalous signal at this site, as detected in the data set collected at the Cu⁺ edge. The metal is presumably shuttled to the identified site via M158 and is then passed on to the high-affinity ion binding site(s) in later E₁ states, likely via C380 and C382. We note that M711 has adopted a different conformation in the E₁ structure, pointing towards the center of M₄-6, compatible with the formation of a single high-affinity site (Fig. 2a-d). Release to the extracellular space can later take place via the release
pathway detected in-between MA, M2, and M6, then, again in agreement with the MA-M2 and M3-6 subdomains revealed in this work. Regulation of P₈₆ transport has previously been proposed to be achieved via the HMBDs, which have been shown to interfere with turn-over in copper-deficient conditions. Interestingly, the location of the last two HMBDs in the X. tropicalis ATP7B E2P structure is structurally incompatible with inhibition in our E1 structure due to the arrangement of the A-domain (Supplementary Fig. 16). Hence, it is possible that inhibition occurs in E2 states, with one HMBD binding in-between the A- and the P-domains, and that release of the HMBD then activates the transport.

In conclusion, we here present the structure of the model protein AfCopA, a copper-transporting P₈₆-ATPase from A. fulgidus, in an inward-facing E1 conformation. In this state, the A-domain arrangement differs compared to other P₈₆-ATPase subfamilies, suggesting the overall P₈₆-ATPase transport mechanism is less conserved than previously assumed. Our data reveal the mechanism of Cu⁺ uptake from cytosolic chaperones and delivery to the ATPase core, in which the A- and the P-domains, and that release of the HMBD then activates the transport.

**Methods**

**Large-scale expression and purification of AfCopAΔΔC**

AfCopAΔΔC (residues G80-G736) (UniProtKB - O29777) was cloned into pET-22b(+) and expressed in *Escherichia coli* (C43 strain). The cells were cultured in LB medium supplemented with 0.1 mg/mL ampicillin at 100 rpm, 37°C in baffled flasks until the optical density (OD600) reached 0.8 – 1. Next, protein expression was induced through the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the temperature was lowered to 20°C. Following 16 h the cells were harvested by 3 h centrifugation at 185,500 × g. The sample was then loaded on a 5 mL HiTrap Chelating HP column. The protein was eluted in buffer C with increasing imidazole concentration, ending with 5 CV of 500 mM imidazole. Fractions containing AfCopAΔΔC were pooled, and TEV protease was added. After a further 2 h at 4°C, the sample was supplemented with 30 mM imidazole, and solid KCl was added to 500 mM final concentration. The sample was then loaded on a 5 mL Hitrap Chelating HP column (Cytiva), the flow-through containing cleaved AfCopAΔΔC was collected, assessed using SDS-PAGE, and concentrated to approximately 20 mg/mL in a Vivasin concentrator (MWCO = 50 kDa). At this stage, 8–10 mg of purified AfCopAΔΔC were routinely obtained from 1 L of cell culture. 50 mg protein were centrifuged at 20,000 × g for 10 min prior to injection on a 120 mL self-packed Superose™ 6 (Cytiva) prep grade column. The protein was eluted in buffer D (20 mM Tris-HCl pH = 7.6, 80 mM KCl, 20% (v/v) glycerol, 1 mM MgCl₂, 5 mM BME) at 10 mL/min. Fractions containing AfCopAΔΔC were pooled and concentrated to 10 mg/mL in a Vivasin concentrator (MWCO = 50 kDa). The protein was flash-frozen in liquid nitrogen and stored in aliquots at −80°C until further usage. Purification results are shown in Supplementary Fig. 17.

**Crystallization**

Macromolecular crystallization trials were conducted using the HiLiDe (high concentrations of lipid and detergent) approach. A 25 mg/mL
stock solution of 1,2-Dioleoyl-sN-Glycero-3-Phosphocholine (DOPC) (Anatrace) in chloroform was prepared, and a thin lipid film was generated in a glass vial through chloroform evaporation under a nitrogen stream. 10 mg/mL AfCopA protein sample and C12E6 was added to obtain final concentrations of 2.25 mg/mL DOPC and 5 mg/mL C12E6. The sample was incubated for 16 h at 4 °C with gentle stirring. Aggregates were removed through ultracentrifugation at 50,000 × g for 20 min, after which 1 mM CuSO4 was added to the supernatant (no supplementation was made for the apo crystals). Crystals were grown using the hanging drop vapor diffusion method, in 24 well plates containing 400 µL reservoir solution supplemented with 5 mM BME, in drops made of 1 µL protein sample and 1 µL reservoir solution. Crystals diffracting to 3.3 Å were obtained following 10 days in 1.5 M ammonium citrate, 0.1 M MES pH = 5.5. Final data diffracting to 2.8 Å were collected on a crystal for which 5.0 mM 06:0 Lyso PC was used as an additive. All crystals were 2.8 Å were collected on a crystal for which 5.0 mM 06:0 Lyso PC was used as an additive. All crystals were grown using the hanging drop vapor diffusion method, in 24 well plates containing 400 µL reservoir solution supplemented with 5 mM BME, in drops made of 1 µL protein sample and 1 µL reservoir solution. Crystals diffracting to 3.3 – 3.5 Å were obtained following 10 days in 1.5 M ammonium citrate, 0.1 M MES pH = 5.5. Final data diffracting to 2.8 Å were collected on a crystal for which 5.0 mM 06:0 Lyso PC (Avanti) was used as an additive. All crystals were fished using Litho-Loops (Molecular Dimensions) and flash-frozen in liquid nitrogen. Data were collected at Swiss Light Source, the Paul Scherrer Institute, Villigen, Switzerland, beam line X06SA.

Structure determination and analysis
Collected data were processed and scaled using XDS46. Downstream structure determination and refinement was accomplished using Phenix44. Initial phases were obtained in Phaser using molecular replacement, applying the structures of the soluble A- and PN-domains of AfCopA (PDB-IDs 2B8E & 3A1C)20,21 together with the M-domain of LpCopA (PDB-ID 3RFU)16 as search models. Initial model building was accomplished in Coot48, and Rosetta refinement was employed in the early stages of model building. The data enabled modeling of side chains in the A-, P- and M-domains, and side chain conformations in the N-domain were guided using available crystal structures (PDB-ID 3AIC). The final model includes residues S2–736, only lacking 4 residues at the N-terminus of the here exploited truncated form. All structural figures were generated using PyMOL47, using the PyMol APBS electrostatics plugin46 for electrostatic surface representations. To establish the AfCopA-AfCopZ docking model, a homology model of the CxxC-harboring C-terminal part of AfCopZ was generated in SwissModel42 using the E. hirae CopZ structure (PDB-ID 3AIC) as a template. Docking to the determined AfCopA EI structure (high-resolution data set) was performed using pyDockWEB with inputs N157–D159 (AfCopA), C149 + C152 (AfCopZ).

Mutagenesis and point mutant production
Mutant plasmids were generated using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). Point mutants were expressed as the wild type in duplicates of 2 L LB medium per mutant, based on separate transformation colonies. Membrane preparation and purification was performed as described above for the wild type AfCopAΔNAC, but with the following differences: Membranes from 2 L cell culture were solubilized for 2 h in 60 mL buffer B supplemented with 1% DDM. Solubilized material was incubated 1 h at 4 °C with 1 mL loose Ni Sepharose™ resin (Cytiva) equilibrated in buffer C, following which affinity purification was performed using gravity columns. The sample was loaded twice, and the resin was then washed first with 5 mL buffer C, followed by 10 mL buffer C with 50 mM imidazole. The protein was eluted in 5 mL buffer C with 500 mM imidazole, and 1 mg TEV protease was added per sample. On the following day, the sample was incubated with 1 mL fresh Ni resin equilibrated in buffer C for 1 h at 4 °C, and then loaded twice on gravity columns. The flow-through containing purified AfCopAΔNAC mutants was collected and concentrated to 20 mg/mL. All the material (3-10 mg protein depending on the mutation) was injected to a Superose 6 Increase 10/300 GL column (Cytiva) and eluted in buffer D. Mutants were concentrated to >5 mg/mL, flash-frozen in liquid nitrogen and stored at −80 °C until further usage.

Liposome preparation
For liposome preparation, a thin film of 20 mg DOPC was generated through chloroform evaporation of a stock solution under nitrogen stream and placed in a vacuum desiccator for 16 h. All buffers were treated with Chelex® 100 Chelating Resin (Bio-Rad) before usage. The lipid film was rehydrated in 20 mMOPS-KOH pH = 6.8, 100 mM NaCl, 1 mM freshly prepared ascorbic acid, 10 mM freshly prepared cysteine, and placed for 3 × 30 min in a bath sonicator. The sample was exposed to 3 freeze-thaw cycles in liquid nitrogen, and subsequently extruded 11 times through 100 nm filters (Avanti). Liposomes were flash-frozen in liquid nitrogen and stored at −80 °C until use.

In vitro ATPase assay
Liposomes were thawed and destabilized through addition of 0.01% (w/v) DDM for 1 h at 18 °C. Reconstitution was performed at a lipid:protein molar ratio of 20:1 with a final protein concentration of 0.5 mg/mL. Concentrated protein sample and liposomes were mixed with reconstitution buffer (20 mM MOPS-KOH pH = 6.8, 100 mM NaCl, 1 mM ascorbic acid) supplemented with 0.15 mg/mL C12E6 and incubated for 1 h at 4 °C. Detergent was removed through addition of Bio-Beads™ SM-2 resin following 1 h, 2 h and 18 h. Proteoliposomes were collected by centrifugation at 60,000 × g for 1.5 h, and were resuspended in the same reconstitution buffer. The samples were then used for measuring in vitro ATPase activity by employing an assay originally developed by Baginski and colleagues44. Briefly, 0.2 mg/mL proteoliposomes were added to 20 mM MOPS-KOH pH = 6.8, 100 mM NaCl, 1 mM ascorbic acid, 10 mM cysteine and 100 µM CuCl2. The assay was performed at 65 °C and the samples were incubated 10 min before the reaction was started by adding 5 mM ATP (Sigma Aldrich). 30 µL of the respective reaction mixtures were transferred to a 96-well microplate and mixed with an equal volume of ascorbic acid solution (formed by mixing a solution containing 0.17 M ascorbic acid and 0.1 % SDS in 0.5 M HCl with aqueous 28.3 mM ammonium heptamolybdate in a 1:5 ratio, all from Sigma Aldrich) after 5, 12 and 18 min. Color development was stopped through addition of 75 µL of sodium arsenic solution (consisting of 0.068 M trisodium citrate, 0.154 M sodium metaarsenic and 2% v/v glacial acetic acid, all from Sigma Aldrich) following 10 min incubation at 18 °C. After 30 min incubation, the absorbance was measured at 860 nm. The presented data are from at least 5 independent measurements on samples from biological duplicates. As also noted by others12, CopA samples exhibit high background activity in the absence of added copper, which we assume arises from trace levels of residual copper in our samples. Background activity of 30% was subtracted from plotted values (Supplementary Fig. 17). Figures representing ATPase activity measurements were generated using GraphPad Prism.

ICP-MS measurements
Cu+ binding stoichiometry in wild type AfCopA and mutants was determined by ICP-MS analysis. The purified protein stocks were diluted to final 1 mg/mL using a buffer containing 20 mM Tris pH = 7.6, 80 mM KCl, 20% (v/v) glycerol, 3 mM MgCl2, 5 mM BME, 0.15 mg/mL C12E6, 4 Cu2+ equivalents (from a CuCl2 stock in H2O) were added to the protein solutions and incubated at room temperature for 15 min. Excess and unbound copper was removed by injecting the protein solutions through a 5 mL HiTrap desalting column, pre-equilibrated with buffer. The concentration of eluted proteins was determined via a Bradford assay with Bovine Serum Albumin (BSA) as a standard. The eluted protein samples were digested in 8% (w/v) HNO3 (Sigma-Aldrich) at 80 °C for 12 h. The samples were subsequently diluted to adjust the HNO3 concentration to final 3% (w/v), and the copper content in samples were analyzed with an Agilent 7900 ICP mass spectrometer connected to a CETACASK-500 auto-sampler for sample injection. Control experiments were conducted for background correction.
using protein buffer excluding protein. Figures representing ICP-MS measurements were generated using GraphPad Prism.

**Molecular dynamics (MD) simulations**

Two ensembles that differed in the CPC region (denoted as I and II in Fig. 4b) were inserted into DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) membrane patches using the CHARMM-GUI membrane builder. Amino acid residue E457 was protonated as predicted by PROPKA3.1. The systems were solvated in TIP3P water and 150 mM NaCl and energy minimized with a 5,000 step steepest-descent algorithm. The systems were equilibrated with gradual release of position restraints from the water and lipids for a total of 30 ns followed by 250 ns non-restrained production runs. Each protein conformation was also simulated in independent repeat simulations starting from a different set of initial velocities, adding up to a sampling total of 250 ns x 4. A Nose-Hoover temperature coupling was applied using a 250 ns non-restrained production runs. Each protein conformation was energy minimized with a 5,000 step steepest-descent algorithm. The systems were equilibrated with gradual release of position restraints on the protein, lipids and ions followed by 100 ns production simulations.

**Reporting summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The structural coordinates generated in this study have been deposited in the Protein Data Bank under accession codes: 7ROI (E1 in the presence of copper), 7ROH (E1 in the presence of copper with data collected at the copper edge), 7ROG (E1 in the absence of copper). Source data are provided with this paper. All data and materials supporting the findings in the manuscript are available from the corresponding author upon reasonable request. Source data are provided with this paper.

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Author contributions
C.G. and P.G. initiated the project. N.S., C.G. and P.G. contributed to identification of scientific problem, experimental planning, data analysis, interpretation. N.S. and C.G. performed cloning, overproduction and purification for the structural and functional studies, as also assisted by P.L. N.S. accomplished the crystals, NS and CG collected diffraction data, and determined the structure. K.W. provided support for the structure determination. N.S. and C.G. refined the structures. N.S. executed ATPase activity assays. N.A. performed ICP-MS measurements supervised by O.M. F.O. and M.A. performed M.D. simulations. N.S. prepared the figures. N.S., C.G. and P.G. wrote the first draft. All authors commented on the manuscript. C.G. and P.G. supervised the project.

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Correspondence and requests for materials should be addressed to Pontus Gourdon.

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